

**IMPROVED RAPID SUBCLONING
USING SITE-SPECIFIC RECOMBINATION**

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5 FIELD OF THE INVENTION

The invention relates to recombinant DNA technology. In particular, the invention relates to compositions, including vectors, and methods for the rapid subcloning of nucleic acid sequences *in vivo* and *in vitro*.

BACKGROUND OF THE INVENTION

10 Molecular biotechnology has revolutionized the production of protein and polypeptide compounds of pharmacological importance. The advent of recombinant DNA technology permitted for the first time the production of proteins on a large scale in a recombinant host cell rather than by the laborious and expensive isolation of the protein from tissues which may only contain minute quantities of the desired protein

15 (e.g., isolation of human growth hormone from cadaver pituitary). The production of proteins, including human proteins, on a large scale in a heterologous host requires the ability to express the protein of interest in the heterologous host. This process typically involves isolation or cloning of the gene encoding the protein of interest followed by transfer of the coding region into an expression vector that contains

20 elements (e.g., promoters) which direct the expression of the desired protein in the heterologous host cell. The most commonly used means of transferring or subcloning a coding region into an expression vector involves the *in vitro* use of restriction endonucleases and DNA ligases. Restriction endonucleases are enzymes which generally recognize and cleave a specific DNA sequence in a double-stranded DNA molecule. Restriction enzymes are used to excise the coding region from the cloning vector and the excised DNA fragment is then joined using DNA ligase to a suitably cleaved expression vector in such a manner that a functional protein may be expressed.

The ability to transfer the desired coding region to an expression vector is often limited by the availability or suitability of restriction enzyme recognition sites. Often multiple restriction enzymes must be employed for the removal of the desired coding region and the reaction conditions used for each enzyme may differ such that it is necessary to perform the excision reactions in separate steps. In addition, it may be necessary to remove a particular enzyme used in an initial restriction enzyme reaction prior to completing all restriction enzyme digestions; this requires a time-consuming purification of the subcloning intermediate. Ideal methods for the subcloning of DNA molecules would permit the rapid transfer of the target DNA molecule from one vector to another *in vitro* or *in vivo* without the need to rely upon restriction enzyme digestions.

SUMMARY OF THE INVENTION

The present invention provides reagents and methods which comprise a system for the rapid subcloning of nucleic acid sequences *in vivo* and *in vitro* without the need to use restriction enzymes.

The present invention provides a method for the recombination of nucleic acid constructs, comprising: providing a first nucleic acid construct comprising, in operable order, an origin of replication, a first sequence-specific recombinase target site, and a nucleic acid of interest, a second nucleic acid construct comprising, in operable order, an origin of replication, a regulatory element and a second sequence-specific recombinase target site adjacent to and downstream from the regulatory element, and a site-specific recombinase; contacting the first and the second nucleic acid constructs with the site-specific recombinase under conditions such that the first and second nucleic acid constructs are recombined to form a third nucleic acid construct, wherein the nucleic acid of interest is operably linked to the regulatory element. The present invention contemplates the use of any type of regulatory element. In some embodiments of the present invention, the regulatory element comprises a promoter element, a fusion peptide (*e.g.*, an affinity domain), or an epitope tag. In preferred embodiments, the nucleic acid of interest comprises a gene.

In some embodiments, the first nucleic acid construct further comprises a selectable marker. In other embodiments, the second nucleic acid construct further comprises a selectable marker. The present invention contemplates that the first and second nucleic acid constructs both comprise selectable markers. In preferred embodiments the selectable markers of the first and second nucleic acid constructs are different from one another. Selectable markers include, but are not limited to a kanamycin resistance gene, an ampicillin resistance gene, a tetracycline resistance gene, a chloramphenicol resistance gene, a streptomycin resistance gene, a spectinomycin resistance gene, the *aadA* gene, the Φ X174 *E* gene, the *strA* gene, and the *sacB* gene.

In preferred embodiments, the first nucleic acid construct further comprises a prokaryotic termination sequence. Prokaryotic termination sequences include, but are not limited to the T7 termination sequence. In other preferred embodiments, the first nucleic acid construct further comprises a eukaryotic polyadenylation sequence. Polyadenylation sequences include, but are not limited to, the bovine growth hormone polyadenylation sequence, the simian virus 40 polyadenylation sequence, and the Herpes Simplex virus thymidine kinase polyadenylation sequence. In yet other preferred embodiments, the first nucleic acid construct further comprises a conditional origin of replication.

In preferred embodiments of the present invention, the first and second sequence-specific recombinase target sites are selected from the group consisting of *loxP*, *loxP2*, *loxP3*, *loxP23*, *loxP511*, *loxB*, *loxC2*, *loxL*, *loxR*, *lox Δ 86*, *lox Δ 117*, *frt*, *dif*, *loxH* and *att*. The present invention contemplates that the first and second sequence-specific recombinase target sites may comprise the same sequence or may comprise different sequences.

In yet other embodiments of the present invention, the first nucleic acid construct further comprises a polylinker.

The present invention contemplates that the recombination methods can be used *in vitro* and *in vivo*. In some *in vivo* embodiments, the site-specific recombinase is

provided by a host cell expressing the site-specific recombinase. In some *in vivo* methods, the contacting of the first and the second nucleic acid constructs with the site-specific recombinase comprises introducing the first and said second nucleic acid constructs into a host cell under conditions such that the third nucleic acid construct is capable of replicating in the host cell.

The present invention further provides methods for precise transfer of nucleic acid molecules by recombination. In some embodiments, the first nucleic acid construct further comprises a third sequence-specific recombinase target site and said second nucleic acid constructs further comprises a fourth sequence-specific recombinase target site. In preferred embodiments, the first sequence-specific recombinase and the third sequence-specific recombinase in the first nucleic acid construct are located on opposite sides of the nucleic acid of interest. It is contemplated that the first and third sequence-specific recombinase target sites are contiguous with, adjacent to, or distant from the nucleic acid of interest. In particularly preferred embodiments the third and fourth sequence-specific recombinase target sites are selected from the group consisting of RS sites and Res sites, although other target sites are contemplated by the present invention. In some embodiments of the this method of the present invention, the first nucleic acid construct further comprises a third sequence-specific recombinase target site and the second nucleic acid constructs further comprises a fourth sequence-specific recombinase target site, wherein the method further comprises providing a second site-specific recombinase and the step of contacting the third nucleic acid construct with the second site-specific recombinase under conditions such that the third nucleic acid construct is recombined to form a fourth and a fifth nucleic acid construct.

The present invention also provides a recombined nucleic acid construct prepared according to any of the above methods.

The present invention further provides a method for the recombination of nucleic acid constructs, comprising: providing a vector, a linear nucleic acid molecule comprising a sequence complementary to at least a portion of said vector, and an *E. coli* host cell, wherein said host cell comprises an endogenous recombination system, a

loss of function *rec* mutation, a suppressor, and a loss of function endogenous restriction modification system mutation; and introducing the vector and the linear nucleic acid molecule into the host cell under conditions such that the linear nucleic acid molecule and the vector are recombined to form a recombinant nucleic acid construct. In preferred embodiments the loss of function *rec* mutation is selected from the group consisting of *recBC* and *recD*. In other preferred embodiments, the suppressor comprises *sbc*. In yet other preferred embodiments, the loss of function endogenous restriction modification system mutation comprises *hsdR*.

The present invention further provides a method for generating a nucleic acid fusion on the 3' end of the nucleic acid of interest in the first nucleic acid construct from above, comprising: providing a tagged linear nucleic acid sample comprising a tag to be added to the 3' end of the nucleic acid of interest, and a sequence complementary to a region of the first nucleic acid construct that is 3' of the nucleic acid of interest; and a host cell capable of endogenous homologous recombination of complementary nucleic acid molecules; and introducing the tagged linear nucleic acid sample and the first nucleic acid construct into the host cell under conditions such that the tagged linear nucleic acid sample and the first nucleic acid construct are recombined to form a tagged nucleic acid construct.

The present invention further provides a method for the cloning of nucleic acid libraries, comprising: providing a plurality of first nucleic acid constructs comprising, in operable order, an origin of replication, a first sequence-specific recombinase target site, and a nucleic acid member from a nucleic acid library, a plurality of second nucleic acid construct comprising, in operable order, an origin of replication, a regulatory element and a second sequence-specific recombinase target site adjacent to and downstream from the regulatory element, and a site-specific recombinase; contacting the plurality of first and second nucleic acid constructs with the site-specific recombinase under conditions such that the plurality of first and second nucleic acid constructs are recombined to form a plurality of third nucleic acid constructs, wherein the nucleic acid members from the nucleic acid library are operably linked to the

regulatory elements. The present invention further provides a nucleic acid library prepared according to the above method.

The present invention also provides a method for the directional cloning of a nucleic acid molecule, comprising: providing first and second portions of a regulatory element, a first nucleic acid molecule comprising the first portion of the regulatory element; and a second nucleic acid molecule comprising the second portion of the regulatory element; and combining the first and the second nucleic acid molecules to produce a third nucleic acid molecule under conditions whereby an intact regulatory element is produced from the combination of the first and the second portions of the regulatory element, wherein the presence of the intact regulatory element in the third nucleic acid molecule indicates a direction of cloning of the first nucleic acid molecule with respect to the second nucleic acid molecule.

The present invention also provides a method for the directional cloning of a nucleic acid molecule, comprising providing: the nucleic acid molecule to be cloned, a first primer comprising sequence complementary to the nucleic acid molecule, a second primer comprising sequence complementary to the nucleic acid molecule and sequence corresponding to a first portion of a *lacO* site, amplification means, and a target nucleic acid molecule comprising a second portion of the *lacO* site; amplifying the nucleic acid molecule with the first and second primers to produce a modified nucleic acid molecule comprising the first portion of a *lacO* site; and ligating the modified nucleic acid molecule into the target nucleic acid such that, when cloned in the desired direction, an intact *lacO* site is produced. In some embodiments, the method further comprises the step of detecting the intact *lacO* site. In particularly preferred embodiments, the target nucleic acid molecule comprises pUNI-30.

The present invention further provides a method for regulated recombination in host cells that constitutively express a recombinase, comprising: providing a host cell expressing a recombinase, a first nucleic acid construct comprising an origin of replication, a first site-specific recombinase site, a second site-specific recombinase site that differs in sequence from the first site-specific recombinase site such that the

recombinase will not initiate recombination between the first and second site-specific recombinase sites, and a selectable marker gene between the first and second site-specific recombinase sites, and a second nucleic acid construct comprising an origin of replication, a third site-specific recombinase target site, and a fourth site-specific recombinase target site that differs in sequence from the third site-specific recombinase site such that the recombinase will not initiate recombination between the third and fourth site-specific recombinase sites; and introducing the first and second nucleic acid constructs into the host cell under conditions such that the first and second nucleic acid constructs are recombined. In some embodiments, the method further comprises the step of selecting for a desired recombinant nucleic acid molecule using the selectable marker. In preferred embodiments, the first nucleic acid construct is a Univector. In alternative preferred embodiments, the second nucleic acid construct is a Univector.

The present invention also provides, a nucleic acid construct comprising, in operable order: a conditional origin of replication; a sequence-specific recombinase target site having a 5' and a 3' end; and a unique restriction enzyme site, said restriction enzyme site located adjacent to the 3' end of the sequence-specific recombinase target site. In some embodiments, the construct further comprises a prokaryotic termination sequence. In yet other embodiments, the construct further comprises a eukaryotic polyadenylation sequence. The present invention contemplates the use of any prokaryotic termination sequence and any eukaryotic polyadenylation sequence. In preferred embodiments, the construct further comprises one or more selectable marker genes. Selectable marker genes include, but are not limited to the kanamycin resistance gene, the ampicillin resistance gene, the tetracycline resistance gene, the chloramphenicol resistance gene, the streptomycin resistance gene, the *strA* gene, and the *sacB* gene. In preferred embodiments, the sequence-specific recombinase target site is selected from the group consisting of *loxP*, *loxP2*, *loxP3*, *loxP23*, *loxP511*, *loxB*, *loxC2*, *loxL*, *loxR*, *loxΔ86*, *loxΔ117*, *frt*, *dif*, *loxH* and *att*.

In some embodiments the construct further comprises a gene of interest inserted into the unique restriction enzyme site. In particular embodiments, the construct has the nucleotide sequence set forth in SEQ ID NO:1 (Figure 26A). In other

embodiments, the construct further comprises a second sequence-specific recombinase target site. In preferred embodiments, the second sequence-specific recombinase target site is selected from the group consisting of RS site and a Res site. In yet other embodiments, the construct further comprises a polylinker.

The present invention further provides a nucleic acid construct comprising in 5' to 3' operable order: an origin of replication; a promoter element having a 5' and a 3' end; and a sequence-specific recombinase target site having a 5' and a 3' end. In some embodiments, the construct further comprises a selectable marker gene.

The present invention also provides a nucleic acid construct comprising in operable order: a promoter element having a 5' and a 3' end; a first sequence-specific recombinase target site having a 5' and a 3' end, wherein the 3' end of the promoter element is located upstream of the 5' end of the sequence-specific recombinase target site; a gene of interest joined to the 3' end of the sequence-specific recombinase target site such that a functional translational reading frame is created; a conditional origin of replication; a first selectable marker gene; a second sequence-specific recombinase target site; and an origin of replication. In some embodiments, the construct further comprises a second selectable marker gene.

The present invention also provides a method for the recombination of nucleic acid constructs, comprising: providing a first nucleic acid construct comprising a *loxH* site, a second nucleic acid construct comprising a *loxH* site; and a site-specific recombinase; and contacting the first and the second nucleic acid constructs with the site-specific recombinase under conditions such that the first and second nucleic acid constructs are recombined. The present invention also provides a recombined nucleic acid construct prepared according to the above method.

DESCRIPTION OF THE DRAWINGS

Figure 1 provides a schematic illustrating certain elements of the pUNI vectors and the Univector Fusion System.

Figure 2A provides a schematic map of the pUNI-10 vector; the locations of selected restriction enzyme sites are indicated and unique sites are indicated by the use of bold type.

5 Figure 2B shows the DNA sequence of the *loxP* site and the polylinkers contained within pUNI-10 (*i.e.*, nucleotides 401-530 of SEQ ID NO:1).

Figure 3A shows the oligonucleotides (SEQ ID NOS:4 and 5) which were annealed to insert a *loxP* site into the polylinker of pGEX-2TKcs to create pGst-*lox*.

10 Figure 3B provides a schematic map of pGEX-2TKcs which includes an enlargement of the multiple cloning site (MCS).

Figure 4A shows the oligonucleotides (SEQ ID NOS:6 and 7) which were annealed to insert a *loxP* site into the polylinker of pVL1392 to create pVL1392-*lox*.

15 Figure 4B provides a schematic map of pVL1392 which includes an enlargement of the multiple cloning site (MCS); the ampicillin resistance gene (Ap^R) and the *tac* promoter (P_{tac}) are indicated.

Figure 5A shows the oligonucleotides (SEQ ID NOS:8 and 9) which were annealed to insert a *loxP* site into the polylinker of pGAP24 to create pGAP24-*lox*.

20 Figure 5B provides a schematic map of pGAP24 which includes an enlargement of the multiple cloning site (MCS); the ampicillin resistance gene (Ap^R), the GAP promoter (P_{GAP}), the origin from the 2μm circle (2μ) and the *TRP1* gene, encoding N-(5'-phosphoribosyl)-anthranilate synthetase, (TRP1) are indicated.

Figure 6A shows the oligonucleotides (SEQ ID NOS:8 and 9) which were annealed to insert a *loxP* site into the polylinker of pGAL14 to create pGAL14-*lox*.

25 Figure 6B provides a schematic map of pGAL14 which includes an enlargement of the multiple cloning site (MCS); the ampicillin resistance gene (Ap^R), the GAL promoter (P_{GAL}), the yeast centromeric sequences (CEN), yeast autonomous replication sequences (ARS) and the *TRP1* gene (TRP1) are indicated.

Figure 7 shows a Coomassie blue-stained SDS-PAGE gel showing the purification of Gst-Cre from *E. coli* cells containing pQL123.

Figure 8 provides a schematic showing the strategy employed for the *in vitro* recombination of a pUNI vector ("pA," pUNI-5) with a pHOST vector ("pB," pQL103) to create a fused construct ("pAB"). The relevant markers on each construct are indicated, as are selected restriction enzyme sites.

5 Figure 9A provides a schematic showing the starting constructs (pUNI-Skp1 and pGst-*lox*) and the predicted fusion construct (pGst-Skp1) generated by an *in vitro* fusion reaction.

10 Figure 9B provides an ethidium bromide-stained gel showing the separation of restriction fragments generated by the digestion of pUNI-Skp1, pGst-*lox* and pGst-Skp1.

Figure 10A shows a Coomassie blue-stained SDS-PAGE gel showing the expression of the Gst-Skp1 protein from *E. coli* cells containing pGst-Skp1.

15 Figure 10B shows a Western blot of an SDS-PAGE gel containing extracts prepared from *E. coli* cells containing pGst-Skp1 which was probed using an anti-Skp1 antibody.

Figure 11 shows a Western blot of an SDS-PAGE gel containing extracts prepared from *E. coli* cells (QLB4) containing either a conventionally constructed Gst-Skp1 plasmid or pGst-Skp1 (produced by an *in vitro* fusion reaction).

20 Figure 12 provides a schematic illustrating the *in vivo* gene trap method for the recombination of *lox*-containing vectors in a host cell constitutively expressing the Cre protein.

Figure 13 provides the nucleotide sequence of the wild-type *loxP* site (SEQ ID NO:12), the *loxP2* site (SEQ ID NO:13), the *loxP3* site (SEQ ID NO:14) and the *loxP23* site (SEQ ID NO:15).

25 Figure 14 shows a schematic for one embodiment of Cre-mediated plasmid fusion.

Figure 15 shows data demonstrating the efficiency of Gst-Cre recombinase activity as measured by UPS.

Figure 16 shows the protein expression of UPS generated fusion proteins containing *loxP* following separation by SDS-PAGE and (A) staining with Coomassie blue, and (B) immunoblotting with anti-Skp1 antibodies.

5 Figure 17 shows a comparison of expression levels between *loxP* and *loxH* containing constructs.

Figure 18 shows the expression of UPS-derived baculovirus expression constructs in insect cells.

Figure 19 shows immunoblotting with anti-HA antibodies of Hela cells expressing Myc-tagged F-box protein under the control of the CMV promoter.

10 Figure 20 shows a schematic representation of the POT reaction.

Figure 21 shows restriction digestion assays of sample that underwent POT with *SKP1* replacing the *E* gene in pAS2-*E*.

Figure 22 shows a schematic of a method for directional subcloning of nucleic acid samples into a Univector.

15 Figure 23 provides a schematic map of the pUNI-10, pUNI-20, and pUNI-30 vectors.

Figure 24 shows a schematic of a method for producing a tagged recombinant protein.

20 Figure 25 shows a schematic of a gap repair scheme for modification of the 3' end of coding regions using homologous recombination.

Figure 26 shows the sequence for: A) SEQ ID NO:1; B) SEQ ID NO:10; and C) SEQ ID NO:11.

DEFINITIONS

25 To facilitate understanding of the invention, a number of terms are defined below.

As used herein, "a conditional origin of replication" refers to an origin of replication that requires the presence of a functional trans-acting factor (e.g., a replication factor) in a prokaryotic host cell. Conditional origins of replication include, but are not limited to, temperature-sensitive replicons such as rep pSC101^{ts}.

As used herein, the term "origin of replication" refers to an origin of replication that is functional in a broad range of prokaryotic host cells (*i.e.*, a normal or non-conditional origin of replication such as the ColE1 origin and its derivatives).

5 The terms "sequence-specific recombinase" and "site-specific recombinase" refer to enzymes that recognize and bind to a short nucleic acid site or sequence and catalyze the recombination of nucleic acid in relation to these sites.

10 The terms "sequence-specific recombinase target site" and "site-specific recombinase target site" refer to a short nucleic acid site or sequence which is recognized by a sequence- or site-specific recombinase and which become the crossover regions during the site-specific recombination event. Examples of sequence-specific recombinase target sites include, but are not limited to, *lox* sites, *frt* sites, *att* sites and *dif* sites.

15 The term "*lox* site" as used herein refers to a nucleotide sequence at which the product of the *cre* gene of bacteriophage P1, Cre recombinase, can catalyze a site-specific recombination. A variety of *lox* sites are known to the art including the naturally occurring *loxP* (the sequence found in the P1 genome), *loxB*, *loxL* and *loxR* (these are found in the *E. coli* chromosome) as well as a number of mutant or variant *lox* sites such as *loxP511*, *loxΔ86*, *loxΔ117*, *loxC2*, *loxP2*, *loxP3*, *loxP23*, *loxS*, and *loxH*.

20 The term "*frt* site" as used herein refers to a nucleotide sequence at which the product of the *FLP* gene of the yeast 2 μ m plasmid, FLP recombinase, can catalyze a site-specific recombination.

25 The term "unique restriction enzyme site" indicates that the recognition sequence for a given restriction enzyme appears once within a nucleic acid molecule. For example, the *EcoRI* site is a unique restriction enzyme site within the plasmid pUNI-10 (SEQ ID NO:1).

A restriction enzyme site is said to be located "adjacent to the 3' end of a sequence-specific recombinase target site" if the restriction enzyme recognition site is located downstream of the 3' end of the sequence-specific recombinase target site.

The adjacent restriction enzyme site may, but need not, be contiguous with the last or 3' nucleotide comprising the sequence-specific recombinase target site. For example, the *Eco*RI site of pUNI-10 is located adjacent (within 3 nucleotides) to the 3' end of the *loxP* site (see Figure 2B); the *Xho*I, *Nde*I, and *Nco*I sites are also adjacent (*i.e.*, within about 10-150 nucleotides) to the *loxP* site but these sites are not contiguous with the 3' end of the *loxP* site in pUNI-10.

The terms "polylinker" or "multiple cloning site" refer to a cluster of restriction enzyme sites on a nucleic acid construct which are utilized for the insertion and/or excision of nucleic acid sequences such as the coding region of a gene, *lox* sites, etc.

The term "prokaryotic termination sequence" refers to a nucleic acid sequence which is recognized by the RNA polymerase of a prokaryotic host cell and results in the termination of transcription. Prokaryotic termination sequences commonly comprise a GC-rich region that has a twofold symmetry followed by an AT-rich sequence [Stryer, *supra*]. A commonly used prokaryotic termination sequence is the T7 termination sequence. A variety of termination sequences are known to the art and may be employed in the nucleic acid constructs of the present invention including, but not limited to, the T_{INT} , T_{L1} , T_{L2} , T_{L3} , T_{R1} , T_{R2} , T_{6S} termination signals derived from the bacteriophage lambda [*Lambda II*, Hendrix *et al.* Eds., *supra*] and termination signals derived from bacterial genes such as the *trp* gene of *E. coli* [Stryer, *supra*].

The term "eukaryotic polyadenylation sequence" (also referred to as a "poly A site" or "poly A sequence") as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. The poly A signal utilized in an expression vector may be "heterologous" or "endogenous." An endogenous poly A signal is one that is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly A signal is one which is isolated from one gene and placed 3' of another gene. A commonly used heterologous poly A signal is the SV40 poly A signal. The SV40 poly A signal is contained on a 237 bp *Bam*HI/*Bcl*I

restriction fragment and directs both termination and polyadenylation [J. Sambrook, *supra*, at 16.6-16.7]; numerous vectors contain the SV40 poly A signal [*e.g.*, pCEP4, pREP4, pEBVHis (Invitrogen)]. Another commonly used heterologous poly A signal is derived from the bovine growth hormone (BGH) gene; the BGH poly A signal is available on a number of commercially available vectors [*e.g.*, pcDNA3.1, pZeoSV2, pSecTag (Invitrogen)]. The poly A signal from the Herpes simplex virus thymidine kinase (HSV tk) gene is also often used as a poly A signal on expression vectors. Vectors containing the HSV tk poly A signal include the pBK-CMV, pBK-RSV, and pOP13CAT vectors from Stratagene.

As used herein, the terms "selectable marker" or "selectable marker gene" refers to the use of a gene which encodes an enzymatic activity that confers the ability to grow in medium lacking what would otherwise be an essential nutrient (*e.g.*, the *TRP1* gene in yeast cells). In addition, a selectable marker may confer resistance to an antibiotic or drug upon the cell in which the selectable marker is expressed. A selectable marker may be used to confer a particular phenotype upon a host cell. When a host cell must express a selectable marker to grow in selective medium, the marker is said to be a positive selectable marker (*e.g.*, antibiotic resistance genes which confer the ability to grow in the presence of the appropriate antibiotic). Selectable markers can also be used to select against host cells containing a particular gene (*e.g.*, the *sacB* gene which, if expressed, kills the bacterial host cells grown in medium containing 5% sucrose and the Φ X174 *E* gene). Selectable markers used in this manner are referred to as negative selectable markers or counter-selectable markers.

As used herein, the term "vector" is used in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The term "vehicle" is sometimes used interchangeably with "vector." A "vector" is a type of "nucleic acid construct." The term "nucleic acid construct" includes circular nucleic acid constructs such as plasmid constructs, phagemid constructs, cosmid vectors, etc. as well as linear nucleic acid constructs (*e.g.*, λ phage constructs and PCR products). The nucleic acid

construct may comprise expression signals such as a promoter and/or an enhancer (in such a case it is referred to as an expression vector).

The term "expression vector" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

The terms "in operable combination," "in operable order," and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The terms "transformation" and "transfection" as used herein refer to the introduction of foreign DNA into prokaryotic or eukaryotic cells. Transformation of prokaryotic cells may be accomplished by a variety of means known to the art including the treatment of host cells with CaCl_2 to make competent cells, electroporation, etc. Transfection of eukaryotic cells may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics, among other means.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

As used herein, the term "recombinant DNA molecule" as used herein refers to a DNA molecule that comprises segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule that is expressed from a recombinant DNA molecule.

DNA molecules are said to have "5' ends" and "3' ends" because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage. Therefore, an end of an oligonucleotides is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5' of the "downstream" or 3' elements. This terminology reflects the fact that transcription proceeds in a 5' to 3' fashion along the DNA strand. The promoter and enhancer elements that direct transcription of a linked gene are generally located 5' or upstream of the coding region. However, enhancer elements can exert their effect even when located 3' of the promoter element and the coding region. Transcription termination and polyadenylation signals are located 3' or downstream of the coding region.

The 3' end of a promoter element is said to be located upstream of the 5' end of a sequence-specific recombinase target site when (moving in a 5' to 3' direction along the nucleic acid molecule) the 3' terminus of a promoter element (the transcription start site is taken as the 3' end of a promoter element) precedes the 5' end of the sequence-specific recombinase target site. The 3' end of the promoter element may be located adjacent (generally within about 0 to 500 bp) to the 5' end of the sequence-specific recombinase target site. Such an arrangement is used when the pHOST vector is not intended to permit the expression of a translational fusion with the gene of interest donated by a pUNI vector. Alternatively, when the pHOST vector is intended to permit the expression of a translational fusion, the 3' end of the promoter element is located upstream of both the sequences encoding the amino-terminus of a fusion protein and the 5' end of the sequence-specific recombinase target

site. In this case, the 5' end of the sequence-specific recombinase target site is located within the coding region of the fusion protein (*e.g.*, located downstream of both the promoter element and the sequences encoding the affinity domain, such as Gst).

As used herein, the phrase "an oligonucleotide having a nucleotide sequence encoding a gene" refers to a nucleic acid sequence comprising the coding region of a gene or, in other words, the nucleic acid sequence that encodes a gene product. The coding region may be present in either a cDNA, genomic DNA, or RNA form. When present in a DNA form, the oligonucleotide may be single-stranded (*i.e.*, the sense strand) or double-stranded. Suitable control elements such as enhancers/promoters, splice junctions, polyadenylation signals, *etc.* may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript. Alternatively, the coding region utilized in the vectors of the present invention may contain endogenous enhancers/promoters, splice junctions, intervening sequences, polyadenylation signals, *etc.* or a combination of both endogenous and exogenous control elements.

As used herein, the term "regulatory element" refers to a genetic element that controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element that facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements are splicing signals, polyadenylation signals, termination signals, *etc.* (defined *infra*).

Transcriptional control signals in eukaryotes comprise "promoter" and "enhancer" elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription [Maniatis, T. *et al.*, *Science* 236:1237 (1987)]. Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect, and mammalian cells and viruses (analogous control elements, *i.e.*, promoters, are also found in prokaryotes). The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a

limited subset of cell types [for review, see Voss, S.D. *et al.*, *Trends Biochem. Sci.*, 11:287 (1986) and Maniatis, T. *et al.*, *supra* (1987)]. For example, the SV40 early gene enhancer is very active in a wide variety of cell types from many mammalian species and has been widely used for the expression of proteins in mammalian cells [Dijkema, R. *et al.*, *EMBO J.* 4:761 (1985)]. Two other examples of promoter/enhancer elements active in a broad range of mammalian cell types are those from the human elongation factor 1 α gene [Uetsuki, T. *et al.*, *J. Biol. Chem.*, 264:5791 (1989), Kim, D.W. *et al.*, *Gene* 91:217 (1990) and Mizushima, S. and Nagata, S., *Nuc. Acids. Res.*, 18:5322 (1990)] and the long terminal repeats of the Rous sarcoma virus [Gorman, C.M. *et al.*, *Proc. Natl. Acad. Sci. USA* 79:6777 (1982)] and the human cytomegalovirus [Boshart, M. *et al.*, *Cell* 41:521 (1985)].

As used herein, the term "promoter/enhancer" denotes a segment of DNA that contains sequences capable of providing both promoter and enhancer functions (*i.e.*, the functions provided by a promoter element and an enhancer element, see above for a discussion of these functions). For example, the long terminal repeats of retroviruses contain both promoter and enhancer functions. The enhancer/promoter may be "endogenous" or "exogenous" or "heterologous." An "endogenous" enhancer/promoter is one which is naturally linked with a given gene in the genome. An "exogenous" or "heterologous" enhancer/promoter is one which is placed in juxtaposition to a gene by means of genetic manipulation (*i.e.*, molecular biological techniques) such that transcription of that gene is directed by the linked enhancer/promoter.

The presence of "splicing signals" on an expression vector often results in higher levels of expression of the recombinant transcript. Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site [Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York (1989) pp. 16.7-16.8]. A commonly used splice donor and acceptor site is the splice junction from the 16S RNA of SV40.

Eukaryotic expression vectors may also contain "viral replicons" or "viral origins of replication." Viral replicons are viral DNA sequences that allow for the

extrachromosomal replication of a vector in a host cell expressing the appropriate replication factors. Vectors that contain either the SV40 or polyoma virus origin of replication replicate to high copy number (up to 10^4 copies/cell) in cells that express the appropriate viral T antigen. Vectors that contain the replicons from bovine papillomavirus or Epstein-Barr virus replicate extrachromosomally at low copy number (~ 100 copies/cell).

As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

As used herein, the term "gene" means the deoxyribonucleotide sequences comprising the coding region of a structural gene and the including sequences located adjacent to the coding region on both the 5' and 3' ends such that the gene corresponds to the length of the full-length mRNA. The sequences that are located 5' of the coding region and which are present on the mRNA are referred to as 5' non-translated sequences. The sequences that are located 3' or downstream of the coding region and which are present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene that are transcribed into nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript. Introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide. When a gene is altered such that its product is no longer biologically active in a wild-type fashion, the mutation is referred to as a "loss-of-function" mutation. When a gene is altered such that a portion or the entirety of the gene is deleted or replaced, the mutation is referred to as a "knockout" mutation.

In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5' and 3' end of the sequences that are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers that control or influence the transcription of the gene. The 3' flanking region may contain sequences that direct the termination of transcription, post-transcriptional cleavage, and polyadenylation.

As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, recombinant Cre polypeptides are expressed in bacterial host cells (*e.g.*, as a Gst-Cre fusion protein) and the Cre polypeptides are purified by the removal of at least a portion of the host cell proteins; the percent of recombinant Cre polypeptides is thereby increased in the sample.

The term "native protein" is used herein to indicate that a protein does not contain amino acid residues encoded by vector sequences; that is the native protein contains only those amino acids found in the protein as it occurs in nature. A native protein may be produced by recombinant means or may be isolated from a naturally occurring source.

As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (*e.g.*, the Cre protein) joined to an exogenous protein fragment (*e.g.*, the fusion partner which consists of non-Cre protein sequences). The fusion partner may enhance solubility of the protein of interest as expressed in a host cell, may provide an affinity tag to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both, among other desired characteristics. If desired, the fusion protein may be removed from the protein of interest by a variety of enzymatic or chemical means known to the art.

DESCRIPTION OF THE INVENTION

The present invention provides compositions and methods that comprise a system for the rapid subcloning of nucleic acid sequences *in vivo* and *in vitro* without the need to use restriction enzymes. This system is referred to as the Univector Fusion System or Univector Plasmid-fusion System (UPS). The UPS employs site-specific recombination to catalyze plasmid fusion between a Univector (*i.e.*, a plasmid containing a gene of interest) and host vectors containing regulatory information. In some embodiments of the present invention, plasmid fusion events are genetically selected and result in placement of the gene of interest under the control of novel regulatory elements. A second UPS-related method of the present invention allows for the precise transfer of coding sequences alone from a Univector into a host vector. UPS further provides means for the subcloning of entire nucleic acid libraries and the directional cloning of linear nucleic acid molecules (*e.g.*, PCR products).

The UPS offers many advantages over previously available technologies for the manipulation of genes. For example, for a routine analysis of a new gene, it may be desirable to express it in bacteria as a glutathione-S-transferase (Gst) or polyhistidine fusion for purification and antibody production, to fuse it to the DNA-binding domain of *GAL4* or *lexA* for two hybrid analysis, to express it from the T7 promoter to allow generation of a riboprobe or mRNA for *in vitro* transcription and translation, and express it in baculovirus, all in the course of a single study. One might also wish to express the gene under the regulation of different promoters in a variety of organisms or to mark it with different epitope tags to facilitate subsequent biochemical or immunological analysis. All of these manipulations consume significant amounts of time and energy using previous available technologies for two reasons. First, each of the different vectors required for these studies were, for the most part, developed independently and thus contain different sequences and restriction sites for insertion of genes. Therefore, genes must be individually tailored to adapt to each of these vectors. Secondly, the DNA sequence of any given gene varies and can contain internal restriction sites that make it incompatible with particular vectors, thereby

complicating manipulation. The advent of the polymerase chain reaction (PCR) has greatly facilitated the alteration of gene sequences and creation of compatible restriction sites for subcloning purposes. However, the high error rate of thermostable polymerases requires the sequence of each PCR-derived DNA fragment to be verified, a time consuming process.

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The availability of whole genome sequences now provides the opportunity to analyze large sets of genes for both genetic and biochemical properties. The need to perform parallel processing of large gene sets exponentially amplifies the current defects associated with conventional cloning methods. The methods and compositions of the present invention provide a series of recombination-based approaches that significantly reduce the time and effort involved in generating multiple transcriptional and translational fusions for gene analysis and cDNA library construction. The present invention provides a system whereby a gene can be placed under the control of any of a variety of promoters or fused in frame to other proteins or peptides without the use of restriction enzymes. As discussed above, the UPS uses site-specific recombination to fuse two plasmids at a unique sequence adjacent to both a regulatory region and the 5' end of the gene of interest, thereby placing the gene under new regulation. This system, together with the other methods and compositions of the present invention discussed herein, provide a multifaceted approach for the rapid and efficient generation and manipulation of recombinant DNA, thus making possible parallel processing of whole genome sets of coding sequences.

The basis of the UPS is a vector termed the "Univector" or the "pUNI" vector into which sequences encoding a gene of interest (cDNA or genomic) are inserted. The pUNI vector has a sequence-specific recombinase target site, such as a *loxP* site, preceding the insertion site for the gene of interest, a selectable marker gene (this feature is optional) and a conditional origin of replication that is active only in host cells expressing the requisite trans-acting replication factor (this feature is optional). The pUNI vectors are designed to contain a gene of interest but lack a promoter for the expression of the gene of interest. The gene of interest may be cloned directly into the pUNI vector (*i.e.*, the pUNI vector may be used as a cloning vector, particularly

for the cloning of cDNA libraries) or a previously cloned gene of interest may be inserted (*i.e.*, subcloned) into the pUNI vector.

Using a sequence-specific recombinase (*e.g.*, Cre recombinase), a precise fusion of the pUNI vector into a second vector containing another sequence-specific recombinase target site is catalyzed. The second vector, referred to generically as a "pHOST" vector, is a vector (*e.g.*, expression vector) that contains the sequence-specific recombinase target site downstream of regulatory element (*e.g.*, a promoter) contained within the pHOST vector. Following the site-specific recombination event which occurs between the single sequence-specific recombinase target sites located on each vector (*e.g.*, the pUNI vector and the pHOST vector), the two vectors are stably fused in a manner that places the gene of interest under the control of the regulatory element contained within the pHOST vector. When used for transfer into an expression vector, this fusion event also occurs in a manner that retains the proper translational reading frame of the gene of interest.

In some embodiment of the present invention, the fusion or recombination event can be selected for by selecting for the ability of host cells, which do not express a trans-acting replication factor required for replication of a conditional origin contained on the pUNI vector, to acquire a selectable phenotype conferred by the selectable marker gene (if present) on the pUNI vector. In these embodiments, the pUNI vector cannot replicate in cells that do not express the trans-acting replication factor and therefore, unless the pUNI vector has integrated into the second vector that contains a non-conditional origin of replication, pUNI will be lost from the host cell.

The Univector Fusion System allows any number of expression or fusion constructs containing the gene of interest present on the pUNI vector to be made rapidly (*e.g.*, within a single day). Using conventional cloning or subcloning techniques which employ restriction enzyme digestion(s), the production of a single expression vector containing a gene of interest can take several days (*i.e.*, for the design and construction of each expression vector). In contrast, with the methods and compositions of the present invention, once a battery of expression vectors modified to

contain the appropriate sequence-specific recombinase target site is made, a gene of interest can be transferred to any number of expression vectors in an afternoon using the Univector Fusion System. For example, Figure 1 provides a schematic illustrating the straightforward recombination methods of the pUNI vectors and the Univector

5 Fusion System.

The present invention further provides methods and compositions for directional subcloning of PCR fragments and other nucleic acid molecules into Univectors or other vectors and methods and compositions for generation of epitope tags and other fusions at the 3' end of open reading frames using homologous

10 recombination.

In general, UPS can be used to fuse any coding region of interest either with a specific promoter to gain novel transcriptional regulation, with another coding sequence to produce a fusion protein with novel properties (*e.g.*, an epitope tag for immunological detection or a DNA binding domain or transcriptional activation

15 domain for two hybrid analysis), or with any other desired regulatory element. As discussed above, the UPS eliminates the need for restriction enzymes, DNA ligases, and many *in vitro* manipulations required for subcloning. This relieves the constraints on cloning vectors with respect to DNA sequence and size since the UPS reaction is independent of vector size or sequence. Furthermore, the time-consuming processed

20 inherent in conventional cloning such as the identification of a suitable vector, designing a cloning strategy, restriction endonuclease digestion, agarose gel electrophoresis, isolation of DNA fragments, and the ligation reaction is shortened to a 20 minute UPS reaction. Due to the uniform nature of the UPS reaction and its simplicity, dozens of constructs can be made simultaneously by simply using different recipient vectors. In addition, in contrast to restriction enzymes and DNA ligases,

25 recombinases (*e.g.*, Gst-Cre) can be made inexpensively in large quantities. These features will save investigators significant amounts of time and expense.

Together, these methods constitute a comprehensive recombinational strategy for the generation and manipulation of recombinant DNA that can be used for the parallel processing of gene sets, an ability required for genomic analyses.

a) **Conditional Origins of Replication and Suitable Host Cells**

In some embodiments of the present invention, the pUNI vector comprises a conditional origin of replication. Conditional origins of replication are origins that require the presence or expression of a trans-acting factor in the host cell for replication. A variety of conditional origins of replication functional in prokaryotic hosts (*e.g.*, *E. coli*) are known to the art. The present invention is illustrated with, but not limited by, the use of the R6K γ origin, oriR, from the plasmid R6K. The R6K γ origin requires a trans-acting factor, the Π protein supplied by the *pir* gene [Metcalf *et al.* (1996) *Plasmid* 35:1]. *E. coli* strains containing the *pir* gene will support replication of R6K γ origins to medium copy number. A strain containing a mutant allele of *pir*, *pir*-116, will allow an even higher copy number of constructs containing the R6K γ origin (*i.e.*, 15 copies per cell for the wild type versus 250 copies per cell for the mutant). This property may be useful when potentially toxic genes are manipulated, although the chances of expression of a toxic gene are low because, in preferred embodiments of the present invention, the Univector either contains no promoter or contains a promoter driving the *neo* gene which is transcribed in the opposite direction from the gene of interest.

E. coli strains that express the *pir* or *pir*-116 gene product include BW18815 (ATCC 47079; this strain contains the *pir*-116 gene), BW19094 (ATCC 47080; this strain contains the *pri* gene), BW20978 (this strain contains the *pir*-116 gene), BW20979 (this strain contains the *pir* gene), BW21037 (this strain contains the *pir*-116 gene) and BW21038 (this strain contains the *pir* gene) (Metcalf *et al.*, *supra*).

Other conditional origins of replication suitable for use on the pUNI vectors of the present invention include, but are not limited to:

- 25 1) the RK2 *oriV* from the plasmid RK2 (ATCC 37125). The RK2 *oriV* requires a trans-acting protein encoded by the *trfA* gene [Ayres *et al.* (1993) *J. Mol. Biol.* 230:174];
- 2) the bacteriophage P1 *ori* which requires the *repA* protein for replication [Pal *et al.* (1986) *J. Mol. Biol.* 192:275];

- 3) the origin of replication of the plasmid pSC101 (ATCC 37032) which requires a plasmid encoded protein, repA, for replication [Sugiura *et al.* (1992) *J. Bacteriol.* 175: 5993]. The pSC101 *ori* also requires IHF, an *E. coli* protein. *E. coli* strains carrying the *himA* and *himD* (*hip*) mutants (the *him* and *hip* genes encode subunits of IHF) cannot support pSC101 replication [Stenzel *et al.* (1987) *Cell* 49:709];
- 5 4) the bacteriophage lambda *ori* which requires the lambda O and P proteins [*Lambda II*, Hendrix *et al.* Eds., Cold Spring Harbor Press, Cold Spring Harbor, NY (1983)];
- 10 5) pBR322 and other ColE1 derivatives will not replicate in *polA* mutants of *E. coli* and therefore, these origins of replication can be used in a conditional manner [Grindley and Kelley (1976) *Mol. Gen. Genet.* 143:311]; and
- 15 6) replication-thermosensitive plasmids such pSU739 or pSU300 which contain a thermosensitive replicon derived from plasmid pSC101, rep pSC101^{ts} which comprises *oriV* [Mendiola and de la Cruz (1989) *Mol. Microbiol.* 3:979 and Francia and Lobo (1996) *J. Bact.* 178:894]. pSU739 and pSU300 are stably maintained in *E. coli* strain DH5 α (Gibco BRL) at a growth temperature of 30°C (42°C is non-permissive for replication of this replicon).
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Other conditional origins of replication, including other temperature sensitive replicons, are known to the art and may be employed in the vectors and methods of the present invention.

b) Sequence-Specific Recombinases And Target Recognition Sites

25 The precise fusion between the pUNI vector and the expression vector is catalyzed by a site-specific recombinase. Site-specific recombinases are enzymes that recognize a specific DNA site or sequence (referred to herein generically as a "sequence-specific recombinase target site") and catalyze the recombination of DNA in

relation to these sites. Site-specific recombinases are employed for the recombination of DNA in both prokaryotes and eukaryotes. Examples of site-specific recombination include, but are not limited to: 1) chromosomal rearrangements that occur in *Salmonella typhimurium* during phase variation, inversion of the FLP sequence during the replication of the yeast 2 μ m circle, and in the rearrangement of immunoglobulin and T cell receptor genes in vertebrates, 2) integration of bacteriophages into the chromosome of prokaryotic host cells to form a lysogen, and 3) transposition of mobile genetic elements (*e.g.*, transposons) in both prokaryotes and eukaryotes. The term "site-specific recombinase" refers to enzymes that recognize short DNA sequences that become the crossover regions during the recombination event and includes recombinases, transposases, and integrases.

The present invention is illustrated with, but not limited by, the use of vectors containing *lox* sites (*e.g.*, *loxP* sites) and the recombination of these vectors using the Cre recombinase of bacteriophage P1. The Cre protein catalyzes recombination of DNA between two *loxP* sites and is involved in the resolution of P1 dimers generated by replication of circular lysogens [Sternberg *et al.* (1981) Cold Spring Harbor Symp. Quant. Biol. 45:297]. Cre can function *in vitro* and *in vivo* in many organisms including, but not limited to, bacteria, fungi, and mammals [Abremski *et al.* (1983) *Cell* 32:1301; Sauer (1987) *Mol. Cell. Biol.* 7:2087; and Orban *et al.* (1992) *Proc. Natl. Acad. Sci.* 89:6861]. A schematic for one embodiment of Cre-mediated plasmid fusion is shown in Figure 14. In this figure, the Univector, pUNI, is the plasmid into which the gene of interest is inserted and pHOST represents the recipient vector that contains the appropriate transcriptional and/or translational regulatory sequences that will eventually control the expression of the gene of interest. A recombinant expression construct is made through Cre-*loxP*-mediated site-specific recombination that fuses these two plasmids. This *in vitro* reaction generates a dimeric recombinant plasmid in which the gene of interest from pUNI is placed downstream of the promoter present on the host vector. In this example, the recombinant plasmid in Figure 14 can be selected in a *pir*⁻ bacterial strain by selecting Kn^r.

The *loxP* sites may be present on the same DNA molecule or they may be present on different DNA molecules; the DNA molecules may be linear or circular or a combination of both. The *loxP* site consists of a double-stranded 34 bp sequence (SEQ ID NO:12) which comprises two 13 bp inverted repeat sequences separated by an 8 bp spacer region [Hoess *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79:3398 and U.S. Patent No. 4,959,317, the disclosure of which is herein incorporated by reference]. The internal spacer sequence of the *loxP* site is asymmetrical and thus, two *loxP* sites can exhibit directionality relative to one another [Hoess *et al.* (1984) *Proc. Natl. Acad. Sci. USA* 81:1026]. When two *loxP* sites on the same DNA molecule are in a directly repeated orientation, Cre excises the DNA between these two sites leaving a single *loxP* site on the DNA molecule [Abremski *et al.* (1983) *Cell* 32:1301]. If two *loxP* sites are in opposite orientation on a single DNA molecule, Cre inverts the DNA sequence between these two sites rather than removing the sequence. Two circular DNA molecules each containing a single *loxP* site will recombine with one another to form a mixture of monomer, dimer, trimer, etc. circles. The concentration of the DNA circles in the reaction can be used to favor the formation of monomer (lower concentration) or multimeric circles (higher concentration).

Circular DNA molecules having a single *loxP* site will recombine with a linear molecule having a single *loxP* site to produce a larger linear molecule. Cre interacts with a linear molecule containing two directly repeating *loxP* sites to produce a circle containing the sequences between the *loxP* sites and a single *loxP* site and a linear molecule containing a single *loxP* site at the site of the deletion.

The Cre protein has been purified to homogeneity [Abremski *et al.* (1984) *J. Mol. Biol.* 259:1509] and the *cre* gene has been cloned and expressed in a variety of host cells [Abremski *et al.* (1983), *supra*]. Purified Cre protein is available from a number of suppliers (*e.g.*, Novagen and New England Nuclear/DuPont).

The Cre protein also recognizes a number of variant or mutant *lox* sites (variant relative to the *loxP* sequence), including the *loxB*, *loxL* and *loxR* sites which are found in the *E. coli* chromosome [Hoess *et al.* (1982), *supra*]. Other variant *lox* sites include

*loxP*511 [5'-ATAACTTCGTATAGTATACATTTACGAAGTTAT-3' (SEQ ID NO:16); spacer region underlined; Hoess *et al.* (1986), *supra*], and *loxC*2 [5'-ACAAC TTCGTATAATGTATGCTATACGAAGTTAT-3' (SEQ ID NO:17); spacer region underlined; U.S. Patent No. 4,959,317]. Cre catalyzes the cleavage of the *lox* site within the spacer region and creates a six base-pair staggered cut [Hoess and Abremski (1985) *J. Mol. Biol.* 181:351]. The two 13 bp inverted repeat domains of the *lox* site represent binding sites for the Cre protein. If two *lox* sites differ in their spacer regions in such a manner that the overhanging ends of the cleaved DNA cannot reanneal with one another, Cre cannot efficiently catalyze a recombination event using the two different *lox* sites. For example, it has been reported that Cre cannot recombine (at least not efficiently) a *loxP* site and a *loxP*511 site; these two *lox* sites differ in the spacer region. Two *lox* sites which differ due to variations in the binding sites (*i.e.*, the 13 bp inverted repeats) may be recombined by Cre provided that Cre can bind to each of the variant binding sites. The efficiency of the reaction between two different *lox* sites (varying in the binding sites) may be less efficient than between two *lox* sites having the same sequence (the efficiency will depend on the degree and the location of the variations in the binding sites). For example, the *loxC*2 site can be efficiently recombined with the *loxP* site, as these two *lox* sites differ by a single nucleotide in the left binding site.

A variety of other site-specific recombinases may be employed in the methods of the present invention in place of the Cre recombinase. Alternative site-specific recombinases include, but are not limited to:

- 1) the FLP recombinase of the 2 μ plasmid of *Saccharomyces cerevisiae* [Cox (1983) *Proc. Natl. Acad. Sci. USA* 80:4223] which recognizes the *frt* site. Like the *loxP* site, the *frt* site comprises two 13 bp inverted repeats separated by an 8 bp spacer [5'-GAAGTCCTATTCTCTAGAAGTATAGGAACTTC-3' (SEQ ID NO:18); spacer underlined]. The FLP gene has been cloned and expressed in *E. coli* (Cox, *supra*) and in mammalian cells (PCT

International Patent Application PCT/US92/01899, Publication No.: WO 92/15694, the disclosure of which is herein incorporated by reference) and has been purified [Meyer-Lean *et al.* (1987) *Nucleic Acids Res.* 15:6469; Babineau *et al.* (1985) *J. Biol. Chem.* 260:12313; and Gronostajski and Sadowski (1985) *J. Biol. Chem.* 260:12328];

5 2) the Int recombinase of bacteriophage lambda (with or without Xis) which recognizes *att* sites (Weisberg *et al.* In: *Lambda II, supra*, pp. 211-250);

10 3) the xerC and xerD recombinases of *E. coli* which together form a recombinase that recognizes the 28 bp *dif* site [Leslie and Sherratt (1995) *EMBO J.* 14:1561];

15 4) the Int protein from the conjugative transposon Tn916 [Lu and Churchward (1994) *EMBO J.* 13:1541];

15 5) TpnI and the β-lactamase transposons [Levesque (1990) *J. Bacteriol.* 172:3745];

20 6) the Tn3 resolvase [Flanagan *et al.* (1989) *J. Mol. Biol.* 206:295 and Stark *et al.* (1989) *Cell* 58:779];

20 7) the SpoIVC recombinase of *Bacillus subtilis* [Sato *et al.* (1990) *J. Bacteriol.* 172:1092];

25 8) the Hin recombinase [Galsgow *et al.* (1989) *J. Biol. Chem.* 264:10072];

25 9) the Cin recombinase [Hafter *et al.* (1988) *EMBO J.* 7:3991]; and

25 10) the immunoglobulin recombinases [Malynn *et al.* *Cell* (1988) 54:453].

c) **Modification of Expression Vectors**

As discussed above, pUNI vectors are used to transfer a gene of interest into a suitably modified vector via site-specific recombination. The modified vectors or host vectors used in the Univector Fusion System are referred to as pHOST vectors. pHOST vectors are generally expression vectors (*e.g.*, plasmids) which have been modified by the insertion of a sequence-specific recombinase target site (*e.g.*, a *lox*

site). However, the pHOST can comprise any regulatory sequence desired for manipulation of nucleic acids. The presence of the sequence-specific recombinase target site on the pHOST plasmid permits the rapid subcloning or insertion of the gene interest contained within a pUNI vector to generate an expression vector capable of expressing the gene of interest. In some embodiments of the present invention, the pHOST vector may encode a protein domain such as an affinity domain including, but not limited to, glutathione-S-transferase (Gst), maltose binding protein (MBP), a portion of staphylococcal protein A (SPA), a polyhistidine tract, etc. A variety of commercially available expression vectors encoding such affinity domains are known to the art. The affinity domain may be located at either the amino- or carboxy-terminus of the fusion protein. When the pHOST plasmid contains a vector-encoded affinity domain, a fusion protein comprising the vector-encoded affinity domain and the protein of interest is generated when the pUNI and pHOST vectors are recombined.

To generate expression vectors intended to generate transcriptional fusions (*i.e.*, pHOST does not contain a vector-encoded protein domain), a sequence-specific recombinase target site is placed after (*i.e.*, downstream of) the start of transcription in the host vector. This is easily accomplished using synthetic oligonucleotides comprising the desired sequence-specific recombinase target site. In designing the oligonucleotide comprising the sequence-specific recombinase target site, care is taken to avoid introducing an ATG or start codon that might initiate translation inappropriately.

To generate expression vectors intended to generate a fusion protein between a vector-encoded protein domain located at the amino-terminus of the fusion protein and the protein of interest (encoded by the gene of interest contained within the pUNI vector) (*i.e.*, a translational fusion), care is taken to place the sequence-specific recombinase target site in the correct reading frame such that: 1) an open reading frame is maintained through the sequence-specific recombinase target site on pHOST, and 2) the open reading frame in the sequence-specific recombinase target site on pHOST is in frame with the open reading frame found on the sequence-specific

recombinase target site contained within the pUNI vector. In addition, the oligonucleotide comprising the sequence-specific recombinase target site on pHOST is designed to avoid the introduction of in-frame stop codons. The gene of interest contained within the pUNI vector is cloned in a particular reading frame so as to facilitate the creation of the desired fusion protein.

The modification of several expression vectors is provided in the examples below to illustrate the creation of suitable pHOST vectors. At present, approximately 40 pHOST vectors have been generated, including GST expression vectors, yeast *GAL1* expression vectors, mammalian CMV expression vectors, and baculovirus expression vectors. In each case, expression was at or near the levels achieved by conventional cloning. A general strategy for generating any pHOST of interest involves the generation of a linker containing the desired sequence-specific recombinase target site (*e.g.*, a *lox* site such as *loxP* or *loxH*) by annealing two complementary oligonucleotides. The annealed oligonucleotides form a linker having sticky ends that are compatible with ends generated by restriction enzymes whose sites are conveniently located in the parental expression vector (*e.g.*, within a polylinker of the parental expression vector). Thus, any vector can be easily adapted for use with the UPS method.

d) *In Vitro* Recombination

The fusion of a pUNI vector and a pHOST vector is accomplished *in vitro* using a purified preparation of a site-specific recombinase (*e.g.*, Cre recombinase). The pUNI vector and the pHOST vector are placed in reaction vessel (*e.g.*, a microcentrifuge tube) in a buffer compatible with the site-specific recombinase to be used. For example, when a Cre recombinase (native or a fusion protein form) is employed, the reaction buffer may comprise 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 30 mM NaCl and 1 mg/ml BSA. When a FLP recombinase is employed, the reaction buffer may comprise 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 100 µg/ml BSA [Gronostajski and Sadowski, *supra*]. The concentration of the pUNI vector and

the pHOST vector may vary between 100 ng to 1.0 μ g of each vector per 20 μ l reaction volume with about 0.1 μ g of each nucleic acid construct (0.2 μ g total) per 20 μ l reaction being preferred. The concentration of the site-specific recombinase may be titrated under a standard set of reaction conditions to find the optimal concentration of enzyme to be used as described in Example 4.

Following the *in vitro* fusion reaction, a portion of the reaction mixture is used to transform a suitable host cell to permit the recovery and propagation of the fused vectors. In some embodiments of the present invention, the host cell employed will not express the trans-acting factor required for replication of the conditional origin of replication contained within the pUNI vector (or alternatively the host cell will be grown at a temperature which is non-permissive for replication of a temperature sensitive replicon contained within the pUNI vector). The host cells will be grown under conditions that select for the presence of the selectable marker contained within the pUNI vector (*e.g.*, growth in the presence of kanamycin when the pUNI vector contains a kanamycin resistance gene). Plasmid or non-chromosomal DNA is isolated from host cells which display the desired phenotype and subjected to restriction enzyme digestion to confirm that the desired fusion event has occurred.

e) Recombination in Prokaryotic Host Cells

The fusion of a pUNI vector and a pHOST vector may be accomplished *in vivo* using a host cell that expresses the appropriate site-specific recombinase (*e.g.*, Cre recombinase). The host cell may express the recombinase as part of its genome or may be supplied with means for expressing the recombinase (*e.g.*, a recombinase expression vector). In embodiments of the present invention that employ a pUNI vector with a conditional origin of replication, the host cell employed lack the ability to express the trans-acting factor required for replication of the conditional origin of replication (or alternatively the host cell will be grown at a temperature which is non-permissive for replication of a temperature sensitive replicon contained within the pUNI vector).

The pUNI vector and the pHOST vector are cotransformed into the host cell using a variety of methods known to the art (e.g., transformation of cells made competent by treatment with CaCl₂, electroporation, etc.). The cotransformed host cells are grown under conditions that select for the presence of the selectable marker contained within the pUNI vector (e.g., growth in the presence of kanamycin when the pUNI vector contains the kanamycin resistance gene). Plasmid or non-chromosomal DNA is isolated from host cells which display the desired phenotype and subjected to restriction enzyme digestion to confirm that the desired fusion event has occurred.

5 **f) Precise ORF Transfer (POT)**

10 UPS results in the fusion of two plasmids and is suitable for the vast majority of expression needs. In rare cases where the size of the recombinant molecule is limiting (e.g., in the generation of retrovirus or adeno-associated viral [AAV] expression constructs), it might be desirable to transfer only the gene of interest and not the approximately 2 kb remainder of the Univector. To accomplish this, a second 15 recombination event is utilized. In some embodiments of the present invention, this second recombination is catalyzed by the R recombinase [Araki *et al.* (1992) *J. Mol. Biol.* 225:25] that allows a resolution of the UPS generated heterodimer as described in Example 9, although a variety of second recombinases will find use with the present invention (e.g., the Res system). POT function *in vivo* and *in vitro*. It is 20 recommended that POT only be used in those cases where size is a limitation.

25 In some embodiments of the present invention, a standard UPS method is utilized to generate a dimer containing the entire pUNI and pHOST vectors, followed by a reaction with the second recombinase that excises the unwanted portions of the Univector. Alternatively, host cells or reaction conditions can be applied that allow both recombination reactions to occur in a single step (*See Example 9*). Cells containing the desired recombinant product can be selected for by using selectable markers, and/or conditional origins of replication.

g) Generation of 3' Gene Fusions on the Univector

While UPS greatly facilitates the generation of fusion proteins at the N-terminus of the protein of interest, it is often necessary to modify proteins on the C-terminus (e.g., to add an epitope tag). To facilitate this class of modification, the present invention takes advantage of *E. coli*'s endogenous homologous recombination system. It has been shown [Winans *et al.* (1985) *J. Bacteriol.* 161:1219] that *E. coli* strains mutant for *recBC*, but containing a suppressor *sbc*, could take up linear DNA and recombine it onto the *E. coli* chromosome or resident plasmids, much as has been shown for *S. cerevisiae*. *recD* mutants have been shown to behave in a similar manner [Russell *et al.* (1989) *J. Bacteriol.* 171:2609]. However, such systems have not been used for recombinant cloning in *E. coli*. In fact, these systems are incompatible with many cloning protocols, as the endogenous restriction modification systems of the cell would digest the samples to be cloned.

The present invention provides means to overcome these problems and to provide for effective cloning and recombination (e.g., with the UPS). To facilitate recombination onto Univector plasmids, the present invention provides BUN10, a *recBCsbcBhsdR* strain expressing *pir-116*. The *hsdR* mutation prevents restriction of nucleic acid (e.g., PCR amplified DNA) by the endogenous restriction modification system of *E. coli*. In one embodiment of the present invention, this system was tested using a 3xMYC epitope tag and the *SKP1* gene in pUNI-10 as the recipient. pML74, which is pUNI-Amp containing a triple (3x) MYC epitope tag followed by a stop codon, was used as template DNA for PCR amplification with two primers, A and B. Primer A (SEQ ID NO:30) is 71 nt long, the first 50 nt of which correspond to the last 50 nt of the *SKP1* coding region and the last 21 nt, the 3' end of the primer, correspond to the first 21 nt of the DNA encoding the 3xMYC tag. The reading frames of *SKP1* and the 3xMYC tag are in register. Primer B (SEQ ID NO:31) is 22 nt long and recognizes a site on pML74 common to pUNI vectors that begins 367 bp from the polylinker region. Amplification using primers A and B and pML74 as a template generated a fragment of DNA with 50 bp homology to the Univector. This

amplification product was co-transformed with *BamH*I-*Sac*I-cleaved pUNI-SKP1 into BUN10 cells and Kn' transformants were selected and analyzed by restriction mapping. Homologous recombination events are selected because they allow the recircularization of the linearized vector. A schematic representation of this method is provided in Figure 25. Ten percent of Kn' transformants resulted in homologous recombination at the C-terminus of the SKP1 gene to generate a SKP1-3xMYC tag. This experiment demonstrates that homologous recombination in *E. coli* can be used to alter the sequence of genes in 3' regions adjacent to restriction sites.

Furthermore, it is clear that this method is generally applicable to broader cloning strategies. Although the example above describes the use of an amplification product for recombination into the pUNI vector, any nucleic acid sample with sufficient sequence complementarity can be used. Thus, the sample to be inserted could be artificially synthesized or prepared by any other means. Additionally, the recombination event can be designed to occur at any desired location on any desired recipient vector (*i.e.*, is not limited to the production of 3' gene fusions).

h) Method for Directional Subcloning into pUNI Vectors

When cloning blunt ended nucleic acid molecules, such as those generated by thermostable polymerases, it is desirable to have a way of identifying desired recombinant molecules (*e.g.*, vectors containing the insert in a desired orientation). This is of great relevance to the UPS because the initial cloning of genes into pUNI will often utilize PCR amplified material. To facilitate this process, the present invention provides a method for directional subcloning into vectors (*e.g.*, pUNI derivatives) that relies upon the generation of a reconstituted regulatory element from two partial sites located on the fragment to be cloned and the recipient vector, respectively. For example, a linear nucleic acid molecule to be inserted into a vector can be designed with a portion of a promoter at its 3' or 5' ends. The recipient vector is then designed with the remainder of the promoter, arranged such that, when the

cloned fragment is inserted in the desired direction, an intact promoter is reconstituted and provides a means of detecting the successful directional cloning event.

It is clear that a variety of reconstituted regulatory elements can be employed to achieve detectable directional cloning. For example, reconstituted regulatory elements that find use with the present invention include, but are not limited to, promoters, repressors, operators, enhancers, enzyme recognitions sites, selectable markers, and conditional origins of replication, among others. It is also contemplated that the reconstituted regulatory element may comprise a negative selection capability, such that fragments cloned in an undesired orientation reconstitute the regulatory element and are selected against. One skilled in the art will recognize the wide range of regulatory elements and applications that can be applied to this system.

To demonstrate the effectiveness of the above approach, the *lac* operator was employed to direct directional subcloning events. Luria and colleagues observed in the early 1960s that phage carrying the binding site for the *lac* repressor, *lacO*, could induce the expression of the endogenous *lacZ* gene by titrating out a limited number of repressor proteins [Miller and Reznikoff, Eds. (1978) *The Operon*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY] and this was shown to be true when *lacO* was present on high copy number plasmids [Marians *et al.* (1976) *Nature* 263:744; and Heyneker *et al.* (1976) *Nature* 263:748], as illustrated in Figure 22A. Figure 22A shows a schematic representation of normal conditions in the absence of inducer (left diagram) where *lacR* is bound to the *lac* operator sites in front of *lacZ* and represses transcription. In the presence of high copy number plasmid containing the *lacO* sequence (right diagram), LacR repressors are titrated out by binding to plasmid borne *lacO* sites and the endogenous *lacZ* gene is expressed.

This observation was taken advantage of by the methods of the present invention, whereby the 3' half of a *lacO* site was placed on a pUNI vector (*i.e.*, pUNI-30). The *lacO* derivative used was a symmetrical 20 bp site that has a *Eco47III* site at the center. To utilize this method for cloning PCR derived material, primers were made corresponding to the *SKP1* gene. A 10 bp sequence corresponding to the 5' half

of the symmetrical *lacO* sequence (shown in Figure 22B) was added to the 5' end of the 3' primer. Figure 22B shows this strategy, whereby primer A (5') and B (3') are used to amplify the gene of interest. The 5' end of primer B contains a half *lacO* site which subsequently becomes the 3-end of the PCR fragment indicated in the Figure.

5 After ligating the PCR fragment into linearized pUNI-30 containing the other half of *lacO*, an intact *lacO* site is reconstituted and, in Lac⁺ cells, results in induction of endogenous β-galactosidase and production of blue colonies in the presence of X-Gal. The PCR fragment was ligated into *Eco47III*-cleaved pUNI-30 and transformed into BUN10, a Lac⁺ *E. coli* strain, and Kn^r colonies were selected on plates containing X-gal. Plasmids containing *SKP1* in the proper orientation were identified by their dark blue color (shown by arrows in Figure 22C). Reclosure of the vector without insert as well as the presence of the PCR fragment in the incorrect orientation result in the production of white or pale blue colonies. Ten out of 10 dark blue colonies contained *SKP1* in the correct orientation. In particularly preferred embodiments, phosphorylated PCR primers are used. In other preferred embodiments, *Taq* polymerase is used, and the material is preferably treated briefly with T4 polymerase and dNTPs to remove the 3' overhangs generated.

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i) Library Transfer Using UPS

In addition to permitting the rapid transfer of a gene of interest from a particular pUNI vector containing a gene of interest into a pHOST vector, the Univector Fusion System permits the rapid exchange of an entire cDNA library to a variety of expression vectors. This capability to essentially transform one library into many libraries is one of the most significant advances made possible by the UPS methods provided by the present invention. The high efficiency of the *in vitro* UPS reaction (*i.e.*, a minimum of 16.8%) coupled with the extremely high efficiency of modern transformation methods makes possible the conversion of whole cDNA libraries constructed in the Univector into expression libraries without loss of representation. Thus, it is contemplated that single cDNA libraries will be converted

into any of a number of different expression libraries such as those used in the two hybrid systems [Durfee *et al.* (1993) *Gene. & Dev.* 7:55; and Aronheim *et al.* (1997) *Mol. Cell. Biol.* 17:3094], for complementation cloning in yeast [Elledge *et al.* (1991) *Proc. Natl. Acad. Sci.* 88:1731], mammalian expression systems [Okayama and Berg (1982) *Mol. Cell. Biol.* 2:161], etc. Thus, the present invention provides methods such that libraries made for one purpose will no longer need to be remade from scratch when needed in a different context; clones isolated from these libraries are easily converted back into simple Univector plasmids compatible with other pHOST vectors for future analysis.

In these methods, the cDNA library is generated using a pUNI vector as the cloning vector (a pUNI library). The entire library may then be transferred (using either an *in vitro* or an *in vivo* recombination reaction) into any expression vector modified to contain a sequence-specific recombinase target site (*e.g.*, a *lox* site) (*i.e.*, into a pHOST vector). This solves an existing problem in the art, in that there is no way, using existing vector systems, to exchange the inserts in a library made in one expression vector *en masse* (*i.e.*, as an entire library) to a different expression vector. Example 10 provides an illustration of such capabilities using methods of the present invention.

In addition, the sequences contained within a pUNI library can be used to recombine with linear λ constructs (which can then be used to isolate specific genes by complementation of appropriate host cell such as *E. coli* or *S. cerevisiae* mutant cells). For example, UPS is compatible with the λYES series of lambda cloning vectors that use *cre-lox* recombination to convert phage clones into plasmids. These vectors are capable of making extremely large cDNA libraries (*i.e.*, greater than 10^8 recombinants per 100 ng of cDNA) and, unlike plasmid libraries, can be propagated with minimal loss of representation. Further as described in Example 7, the *in vivo* gene trap method, a variation of the Univector Fusion System, can be used to transfer linear DNA fragments that lack a selectable marker, such as a PCR product, into a variety of expression vectors.

An extremely important application of the UPS method is in the manipulation of whole genome sets of coding regions. For organisms whose genomes have been sequenced, a complete set of identified ORFs, or "Unigene" set, can be constructed in the Univector and be systematically converted by UPS into any kind of expression library. Also, the simplicity and uniformity of the UPS reaction makes it readily amenable to automation for systematic conversion of arrayed clones. This greatly expedites the functional characterization of whole genomes and help further the progression of genome projects into proteome projects.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); g (gravitational field); vol (volume); DNA (deoxyribonucleic acid); RNA (ribonucleic acid); kdal or kD (kilodaltons); OD (optical density); EDTA (ethylene diamine tetra-acetic acid); *E. coli* (*Escherichia coli*); SDS (sodium dodecyl sulfate); PAGE (polyacrylamide gel electrophoresis); ts (temperature sensitive); p (plasmid); LB (Luria-Bertani medium: per liter: 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl, pH to 7.5 with NaOH); ml (milliliter); µl (microliter); M (Molar); mM (millimolar); µM (microMolar); g (gram); µg (microgram); ng (nanogram); U (units), mU (milliunits); min. (minutes); sec. (seconds); % (percent); bp (base pair); kb (kilobase); PCR (polymerase chain reaction); Tris (tris(hydroxymethyl)-aminomethane); PMSF (phenylmethylsulfonylfluoride); BSA (bovine serum albumin); IPTG (isopropyl-β-D-thiogalactoside); ORF (open reading frame); ATCC (American Type Culture Collection, Rockville, MD); Bio-Rad (Bio-Rad Corp., Hercules, CA); Invitrogen (Invitrogen, Corp., San Diego, CA); New England Nuclear/Du Pont (Boston, MA); Novagen (Novagen, Inc., Madison, WI); Pharmacia or Pharmacia Biotech (Pharmacia Biotech, Piscataway, NJ); Pharmingen (PharMingen, San Diegi,

CA); Gibco BRL (Gaithersburg, MD); and Stratagene (Stratagene Cloning Systems, La Jolla, CA).

EXAMPLE 1

Construction Of Univector Constructs

5 In this example, illustrative Univector constructs are provided. The map for several Univectors is shown in Figure 23, showing pUNI-10, pUNI-20, and pUNI-30. In this figure, nucleotide positions (in parentheses) of unique restriction enzyme cleavage sites are shown. Functional sequences are shown as filled boxes and are labeled inside of the circle. Boxes with arrows are genes transcribed in the direction 10 of the arrow. Below each map is the sequence of the polylinker region displayed as coding triplets in frame with the open reading frame of *loxP*. Unique restriction enzyme cleavage sites are in bold. General features of these Univectors include a *loxP* site placed adjacent to the 5' end of a polylinker for insertion of cDNAs. *loxP* has a single open reading frame that is in frame with the ATG of the *NdeI* and *NcoI* sites of 15 the polylinker. This facilitates the subsequent generation of protein fusions as noted below. Following the polylinker are bacterial and eukaryotic transcriptional terminators to facilitate 3' end formation of transcripts. The Univectors also comprise a conditional origin or replication derived from R6K γ that allows their propagation only in bacterial hosts expressing the *pir* gene originally from R6K γ [Metcalf *et al.* 20 (1994) *Gene* 138:1]. The Univectors also have the *neo* gene from *Tn5* for selection in bacteria (*e.g.*, selection of recombinant products of UPS is achieved by selecting for kanamycin resistance after transformation into a *pir*⁻ strain because the *neo* gene on the pUNI can only be propagated when covalently linked to an origin or replication that is functional in a *pir*⁻ background). pUNI-20 contains additional site specific 25 recombination sites, such as RS, that facilitate precise ORF transfer (POT), as described below.

One Univector construct, the pUNI-10 vector, contains a *loxP* site, a kanamycin resistance gene (Kn^R) and the R6K γ conditional origin of replication ($\text{OriR}_{\text{R6K}\gamma}$). The $\text{OriR}_{\text{R6K}\gamma}$ is functional only in *E. coli* strains expressing the Π replication protein (*i.e.*, the product of the *pir* gene). A gene of interest is placed within pUNI-10 (either as a result of constructing a library in pUNI-10 or by subcloning a previously cloned gene of interest). Once the gene of interest is contained within pUNI-10, any number of plasmid expression constructs containing this gene of interest can be constructed rapidly (*e.g.*, within a single day). The expression constructs will contain an antibiotic resistance gene other than kanamycin (*e.g.*, ampicillin). Using the site-specific recombinase, Cre, a precise fusion between the pUNI vector and any other *loxP* site-containing vector comprising the desired expression signals adjacent to the *loxP* site is catalyzed. The site-specific recombination event which occurs between the single *loxP* sites located on each plasmid (*e.g.*, pUNI and the expression vector) results in the stable fusion of these two plasmids in such a manner as to place the expression of the gene of interest under the control of the expression signals contained within the expression vector. This subcloning event occurs without the need to use restriction enzymes. The fusion of pUNI-10 and the expression vector is selected for by selecting for the ability of *E. coli* cells that do not express the Π protein to grow in the presence of kanamycin. pUNI cannot replicate in *E. coli* cells that do not express the Π protein unless pUNI has fused or integrated into another plasmid that contains a normal (*i.e.*, not a conditional) origin of replication (*e.g.*, the Col E1 origin). In this case, pUNI will be replicated (as part of the fusion plasmid) and kanamycin resistance will be conferred on the host cell.

a) Generation of pUNI-10

Figure 2A provides a schematic map of the pUNI-10 vector; the locations of selected restriction enzyme sites are indicated (with the exception of *Not*I, all sites shown are unique). Figure 2B shows the DNA sequence of the *loxP* site and the polylinkers contained within pUNI-10 (*i.e.*, nucleotides 401-530 of SEQ ID NO:1).

Nucleotides 1-400 of pUNI-10 contain the conditional origin of replication from R6K γ (OriR_{R6K γ}); the OriR_{R6K γ} was derived from the plasmid R6K (ATCC 37120) [Metcalf *et al.* (1996) Plasmid 35:1]; nucleotides 401-414 comprise a *NotI*-*KpnI* polylinker that facilitates the exchange of *lox* sites; pUNI-10 contains a wild-type *loxP* site (as discussed above, pUNI vectors containing modified *lox* sites may be employed). Nucleotides 415-448 comprise the wild-type *loxP* site; nucleotides 449-527 comprise a polylinker used for the insertion of the gene of interest (genomic or cDNA sequences). Nucleotides 528-750 contain the polyA addition sequence from bovine growth hormone (BGH) (the BGH polyA sequence is available on a number of commercially available vectors including pcDNA3.1 (Invitrogen)); the BGH polyA sequence provides a 3' end for transcripts expressed in mammalian and other eukaryotic cells. The art is aware of other eukaryotic polyA sequences that may be used in place of the BGH polyA sequence (*e.g.*, the SV40 poly A sequence, the TK polyA sequence, etc.). Nucleotides 751-890 contain the T7 terminator sequence which is used to terminate transcription in prokaryotic hosts (numerous prokaryotic termination signals are known to the art and may be employed in place of the T7 terminator sequence). Nucleotides 890-895 comprise an *EcoRV* restriction enzyme recognition site and nucleotides 896-2220 comprise the kanamycin resistance gene (Kan or Kn^R) from Tn5 which provides a positive selectable marker. The Kn^R gene found on pUNI-10 was modified using site-directed mutagenesis to remove the naturally occurring *NcoI* site such that pUNI-10 contains a unique *NcoI* site in the polylinker region located at nucleotides 449-527. pUNI vectors need not contain a Kn^R gene (modified or wild-type); other selectable genes may be used in place of the Kn^R gene (*e.g.*, ampicillin resistance gene, tetracycline resistance gene, zeocinTM resistance gene, etc.). The pUNI vector need not contain a selectable marker, although the use of a selectable marker is preferred. When a selectable marker is present on the pUNI vector, this marker is preferably a different selectable marker than that present on the pHOST vector. The nucleotide sequence of pUNI-10 is provided in SEQ ID NO:1.

EXAMPLE 2

Construction Of Host Plasmids For Use In The Univector Plasmid-Fusion System

Host plasmids used in the Univector plasmid fusion system are referred to as pHOST plasmids. pHOST plasmids or vectors are generally expression vectors that have been modified by the insertion of a site-specific recombination site, such as a *lox* site. The presence of the *lox* site on the pHOST plasmid permits the rapid subcloning or insertion of the gene interest contained within a pUNI vector to generate an expression vector capable of expressing the gene of interest. The pHOST vector may encode a protein domain such as an affinity domain including, but not limited to, glutathione-S-transferase (Gst), maltose binding protein (MBP), a portion of staphylococcal protein A (SPA), a polyhistidine tract, etc. A variety of commercially available expression vectors encoding such affinity domains are known to the art. When the pHOST plasmid contains a vector-encoded affinity domain, a fusion protein comprising the vector-encoded affinity domain and the protein of interest is generated when the pUNI and pHOST vectors are recombined.

In some embodiments of the present invention, the host vector features include the Col E1 origin of replication and the *bla* gene for propagation and selection in bacteria, a *loxP* site for plasmid fusions and a specific promoter residing upstream of, and adjacent to, the *loxP* site. Host vectors may also comprise sequences responsible for propagation, selection, and maintenance in organisms other than *E. coli*.

To generate expression vectors intended to generate transcriptional fusions (*i.e.*, pHOST does not contain a vector-encoded protein domain), a *lox* site is placed after (*i.e.*, downstream of) the start of transcription in the host vector. This is easily accomplished using synthetic oligonucleotides comprising the desired *lox* site. In designing the oligonucleotide comprising the *lox* site, care is taken to avoid introducing an ATG or start codon that might initiate translation inappropriately.

To generate expression vectors intended to generate a fusion protein between a vector-encoded protein domain and the protein of interest (encoded by the gene of

interest contained within the pUNI vector), care is taken to place the *lox* site in the correct reading frame such that 1) an open reading frame is maintained through the *lox* site on pHOST and 2) the open reading frame in the *lox* site on pHOST is in frame with the open reading frame found on the *lox* site contained within the pUNI vector.

5 In addition, the oligonucleotide comprising the *lox* site on pHOST is designed to avoid the introduction of in-frame stop codons. The gene of interest contained within the pUNI vector is cloned in a particular reading frame so as to facilitate the creation of the desired fusion protein.

10 The modification of several expression vectors is provided below to illustrate the creation of suitable pHOST vectors. In each case, the general strategy involved the generation of a linker containing a *lox* site by annealing two complementary oligonucleotides. The annealed oligonucleotides form a linker having sticky ends that are compatible with ends generated by restriction enzymes whose sites are conveniently located in the parental expression vector (*e.g.*, within the polylinker of 15 the parental expression vector).

a) **Modification of the pGEX-2TKcs Prokaryotic Expression Vector**

pGEX-2TKcs is an expression vector active in *E. coli* cells which is designed for inducible, intracellular expression of genes or gene fragments as fusions with Gst. pGEX-2TKcs contains the IPTG-inducible *tac* promoter (P_{tac}) and was derived from pGEX-2TK (Pharmacia Biotech) as follows. The polylinker sequence of pGEX-2TK, 20 5'-GGATCCCCGGGAATTCT-3' (SEQ ID NO:2), was replaced with the following sequence: 5'-GGATCGCATATGCCATGGCTCGAGGATCCGAATTCT-3' (SEQ ID NO:3) to generate the pGEX-2TKcs vector.

A linker containing a *loxP* site was generated by annealing the following 25 oligonucleotides: 5'-CATGGCTATAACTTCGTATAGCATACATTATACGAA GTTATG-3' (SEQ ID NO:4) and 5'-GATCCATAACTTCGTATAATGTATGC TATACGAAGTTATAGC-3' (SEQ ID NO:5). When annealed, these two oligonucleotides form a double-stranded linker having a 5' end compatible with an

*Nco*I sticky end and a 3' end compatible with a *Bam*HI sticky end (Figure 3A). pGEX-2TKcs was digested with *Nco*I and *Bam*HI (Figure 3B) and the annealed *loxP* linker was inserted to form pGst-*lox*.

b) Modification of the pVL1392 Baculovirus Expression Vector

5 pVL1392 is an expression vector that contains the polyhedrin promoter which is active in insect cells (Pharmingen). A linker containing a *loxP* site was generated by annealing the following oligonucleotides: 5'-GGCCGGACGTCTAACTTCGTAT AGCATACTTACGAAGTTATG-3' (SEQ ID NO:6) and 5'-GATCCATAACTCGTATAATGTATGCTATACGAAGTTATGACGTCC-3' (SEQ ID NO:7). When
10 annealed, these two oligonucleotides form a double-stranded linker having a 5' end compatible with a *Not*I sticky end and a 3' end compatible with a *Bam*HI sticky end (Figure 4A). pVL1392 was digested with *Not*I and *Bam*HI (Figure 4B) and the annealed *loxP* linker was inserted to form pVL1392-*lox*.

c) Modification of the pGAP24 Yeast Expression Vector

15 pGAP24 is an expression vector that is based on the yeast 2 μ m circle and contains the constitutive GAP (glyceraldehyde 3-phosphate dehydrogenase) promoter (P_{GAP}) which is active in yeast cells and the *TRP1* gene (used a selectable marker when the cells are grown in medium lacking tryptophan) [the GAP promoter is available on pAB23; Schilds (1990) *Proc. Natl. Acad. Sci. USA* 87:2916]. A linker containing a
20 *loxP* site was generated by annealing the following oligonucleotides: 5'-TCGAGAC GTCATACTTCGTATAGCATACATTATACGAAGTTATGC-3' (SEQ ID NO:8) and 5'-GGCCGCATAACTTCGTATAATGTATGCTATACGAAGTTATGACGTC-3' (SEQ ID NO:9). When annealed, these two oligonucleotides form a double-stranded linker having a 5' end compatible with a *Xho*I sticky end and a 3' end compatible with
25 a *Not*I sticky end (Figure 5A). pGAP24 was digested with *Xho*I and *Not*I (Figure 5B) and the annealed *loxP* linker was inserted to form pGAP24-*lox*.

d) Modification of the pGAL14 Yeast Expression Vector

pGAL14 is a yeast centromeric expression vector that contains the GAL promoter (P_{GAL}), which is induced by the presence of galactose in the medium, and the *TRP1* gene. A linker containing a *loxP* site was generated by annealing together the oligonucleotides listed in SEQ ID NOS:8 and 9. When annealed, these two oligonucleotides form a double-stranded linker having a 5' end compatible with a *XhoI* sticky end and a 3' end compatible with a *NotI* sticky end (Figure 6A). pGAL14 was digested with *XhoI* and *NotI* (Figure 6B) and the annealed *loxP* linker was inserted to form pGAL14-*lox*.

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EXAMPLE 3

Expression And Purification Of A Gst-Cre Fusion Protein

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In order to provide a source of purified Cre recombinase for the *in vitro* recombination of plasmids, the *cre* gene was inserted into a Gst expression vector such that a fusion protein comprising Gst at the amino-terminal end and Cre recombinase at the carboxy-terminal end was produced. The Gst-Cre fusion protein was purified by chromatography using Glutathione Sepharose 4B (Pharmacia). Purified Gst-Cre can be stored at -80°C, -20°C, or 4°C for several months without significant loss of activity.

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To simplify Cre purification, a plasmid expressing a GST-*cre* fusion protein was constructed, pQL123. The *cre* gene was isolated by polymerase chain reaction (PCR) amplification using the plasmid pBS39 (U.S. Patent 4,959,317). U.S. Patent Nos. 4,683,195, 4,683,202 and 4,965,188 describe PCR methodology and are incorporated herein by reference. The primers used in the PCR were designed to introduce an *NcoI* site at the first ATG in the *cre* open reading frame. The PCR product was cloned into a TA cloning vector (pCRII.1; Invitrogen) and then was subcloned as an *NcoI-EcoRI* fragment into pGEX-2TKcs (Example 2) to generate pQL123. The ligation products were used to transform DH5 α cells and the desired recombinant was isolated and used to transform BL21(DE3) cells (Invitrogen).

The nucleotide sequence of the Gst-Cre coding region within pQL123 is listed in SEQ ID NO:10 (Figure 26B). The amino acid sequence of the fusion protein expressed by pQL123 is listed in SEQ ID NO:11 (Figure 26C).

To express the Gst-Cre fusion protein, BL21(DE3) cells containing the pQL123 plasmid were grown at 37°C in LB containing 100 µg/ml ampicillin until the OD₆₀₀ reached 0.6. Expression of the fusion protein was then induced by the addition of IPTG to a final concentration of 0.4 mM and the cells were allowed to grow overnight at 25°C. Following induction, the bacterial cells were pelleted by centrifugation at 5,000 x g at 4°C and the supernatant was discarded. A cell lysate was prepared as follows. Cells harvested from 0.5 liter of culture were suspended in 35 ml of a solution containing 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 5 µg/ml of each of leupeptin, antipain, aprotinin and 1 mM PMSF at 4°C. The cells were incubated for 10 min on ice and then disrupted by sonication (3 x 15 sec bursts) using a sonicator (Ultrasonic Heat Systems Model 200R) at full power. The lysate was then clarified by centrifugation at 12,000 rpm using a SS34 rotor (Sorvall).

The Gst-Cre fusion protein was affinity purified from the cell lysate by chromatography on Glutathione Sepharose 4B (Pharmacia) according to the manufacturer's instructions. The protein concentration of Gst-Cre was determined by Bradford analysis (BioRad).

Aliquots of the cell lysate before and after chromatography on Glutathione Sepharose 4B were applied to an SDS-PAGE gel. Following electrophoresis, the gel was stained with Coomassie blue. The stained gel is shown in Figure 7. In Figure 7, lanes 1 and 2 contain the cell lysate before and after chromatography, respectively. The arrowhead indicates the Gst-Cre fusion protein. The migration of the molecular weight protein markers is indicated to the left of lane 1. The results shown in Figure 7 demonstrate the purification of the Gst-Cre fusion protein. This fusion protein was shown to be functional (*i.e.*, capable of mediating recombination between *lox* sites) in the *in vitro* recombination assay described below.

Gst-Cre retained high recombinase activity as measured by UPS. The efficiency of this reaction reached up to 16.8% as shown in Figure 15, similar to that for native Cre (Abremski *et al.*, *supra*). In this figure, the indicated amounts of Gst-Cre were incubated with pUNI-10 and pQL103 plasmid DNA as described below.

- 5 Percentage of recombinants were calculated by measuring the ratio of total kanamycin resistant transformants (fusion events between pUNI-10 and pQL103) relative to total ampicillin resistant transformants (pQL103 alone and pUNI-10-pQL103 fusions). The efficiency of Gst-Cre was examined in a second reaction producing a tagged recombinant protein as diagrammed in Figure 24, fusing a Gst tag to Skp1.
- 10 Recombinant plasmids isolated from Kn^r transformants were shown by restriction analysis to be correct fusion products between the Univector and the host vector via the *loxP* sites. In this case, 10 of 12 Kn^r transformants were the correct heterodimer (Figure 9) and 2 were trimers (Figure 9, lanes 8 and 10) with two copies of pUNI fused to a host vector. It should be noted that trimeric plasmids also have a correct fusion junction that places the gene of interest adjacent to the desired regulatory sequences and are fully functional for most needs. However, the isolation of trimeric plasmids can be nearly eliminated if gel purified monomeric supercoiled host DNA is used. This method is highly efficient and typically requires only one or two minipreps to identify the desired construct.
- 15

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EXAMPLE 4

In Vitro Recombination Using The Univector Plasmid Fusion System

- The Univector Plasmid Fusion System permits the *in vitro* recombination of two plasmids. Figure 8 provides a schematic showing the strategy employed for *in vitro* recombination. pA represents a generic pUNI vector that contains a *loxP* site, a kanamycin resistance gene and the conditional R6K origin that is only functional in *E. coli* strains expressing the Π protein (*e.g.*, *E. coli* strains BW18815, BW19094, BW20978, BW20979, BW21037, BW21038). pB represents a generic pHOST vector
- 25

that contains a *loxP* site, an ampicillin resistance gene and a Col E1 origin of replication. pAB represents the fused plasmid which results from the Cre-mediated fusion of pA and pB.

To illustrate the *in vitro* recombination reaction, pUNI-5 (a pUNI vector which differs from pUNI-10 only in that pUNI-5 retains the *Nco*I site in the Kn^R gene and contains a different polylinker) was employed as pA and pQL103, an ampicillin-resistant plasmid containing a *loxP* site and the ColE1 origin, was employed as pB. In a total reaction volume of 20 µl, 0.2 µg of each pUNI-5 (pA) and pQL103 (pB) were mixed in a buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 30 mM NaCl and 1 mg/ml BSA. The amount of purified Gst-Cre (Example 3) was varied from 0 to 1.0 µg. The reactions were incubated at 37°C for 20 minutes and then the reactions were placed at 70°C for 5 min. to inactivate the Gst-Cre protein. Five microliters of each reaction mixture were used directly to transform competent DH5 α cells (CaCl₂ treated). The transformed cells were plated onto LB/Amp (100 µg/ml amp) and LB/Kan (40 µg/ml kan) plates and the number of ampicillin resistant (Ap^R) and kanamycin-resistant (Kn^R) colonies were counted. The results are summarized in Table 1.

TABLE 1

| Gst-Cre (µg/reaction) | Ap ^R Colonies | Kn ^R Colonies | % of Total Kn ^R /Ap ^R |
|-----------------------|--------------------------|--------------------------|---|
| 0 | 2.6 x 10 ⁴ | 0 | 0 |
| 0.01 | 1.9 x 10 ⁴ | 571 | 3 |
| 0.05 | 1.1 x 10 ⁴ | 682 | 6.2 |
| 0.1 | 1.5 x 10 ⁴ | 502 | 3.3 |
| 0.5 | 0.3 x 10 ⁴ | 104 | 3.4 |
| 1.0 | 0.3 x 10 ⁴ | 52 | 1.7 |

The results shown in Table 1 demonstrate, that under these reaction conditions 0.05 µg purified Gst-Cre per 20 µl reaction yields the most efficient rate of plasmid fusion. Plasmid DNA was isolated from individual kanamycin-resistant colonies (using standard mini-prep plasmid DNA isolation protocols) and subjected to

restriction enzyme digestion to determine the structure of the fused plasmids. This analysis revealed that plasmid DNA isolated from the kanamycin-resistant colonies represented a dimer created by the desired fusion of pUNI-5 and pQL103 via the *loxP* sites. These results demonstrate that the Univector Plasmid Fusion System can be used
5 to rapidly fuse two plasmids together *in vitro*.

EXAMPLE 5

In Vitro Fusion Between A pUNI Vectors Containing
Genes Of Interest And *Lox*-Containing Expression Vectors
Produces Fused Vectors Capable Of Expressing The Gene Of Interest

10 In Example 4 it was demonstrated that the Univector Plasmid Fusion System can be used to rapidly fuse two plasmid constructs together *in vitro*. In this example, the ability of the Univector Plasmid Fusion System to fuse two plasmids together in a manner that places the gene of interest contained on the pUNI vector under the transcriptional control of a promoter contained on the pHOST or expression vector in
15 such a manner that a functional protein of interest is expressed from the fused construct. A series of expression plasmids were made by UPS and tested for expression in several contexts.

a) Insertion Of A Gene Of Interest Into The pUNI-10 Vector

20 The cDNA encoding the wild-type yeast Skp1 protein [Bai *et al.* (1996) *Cell* 86:263] was cloned into the pUNI-10 vector between the *Nde*I and *Bam*HI sites to generate pUNI-Skp1; the yeast *SKP1* cDNA sequence is available as GenBank Accession No. U61764. Skp1 is an essential protein involved in the regulation of the cell cycle in yeast. Yeast cells containing a temperature sensitive mutant of Skp1 cannot grow at the non-permissive temperature (37°C).

b) ***In Vitro* Fusion Reactions And Complementation Assays**

pUNI-Skp1 was recombined with pGAP24-*lox* (Example 2) and pGAL14-*lox* (Example 2) using the *in vitro* reaction described in Example 4; 0.2 µg of Gst-Cre was used per 20 µl reaction. The resulting plasmid fusions were termed pGAP24-Skp1 and pGAL14-Skp1. pGAP24-Skp1 and pGAL14-Skp1 were then transformed into the temperature sensitive (ts) *skp1-11* mutant yeast strain Y555 (Bai *et al.*, *supra*) and the transformed yeast cells were plated onto SC-tryptophan plates (to select for the expression of the selectable marker *TRP1*) and incubated at either a permissive (25°C) or non-permissive temperature (37°C). The plates which received yeast cells transformed with pGAL14-Skp1 contained galactose. The ability of the transformed cells to grow at the non-permissive temperature is dependent upon the expression of the wild-type *skp1* gene encoded by a properly fused pUNI-Skp1/expression vector construct. As a control, the yeast *SKP1* genomic clone contained in a *URA3 CEN* vector (produced by conventional cloning techniques) was used to transform the ts *skp1-11* mutant yeast strain Y555 and the transformed cells were also plated at 25°C and 37°C. In each case, an expression vector (*e.g.*, pRS414 or pRS415; Bai *et al.*, *supra*) lacking the *SKP1* gene but containing the same selectable marker (*i.e.*, *TRP1*) as either pGAP24-Skp1, pGAL14-Skp1 or *URA3 CEN*-Skp1 was used to transform Y555 cells as a control capable of permitting the growth of transformed Y555 cells on selective medium at the permissive temperature.

The results demonstrated that the *URA3 CEN-SKP1* construct produced by conventional cloning techniques produced a functional Skp1 protein which was capable of complementing the lethality of the *skp1-11* ts mutation. More importantly, the results demonstrated that the *in vitro* fusion reaction that created pGAP24-Skp1 and pGAL14-Skp1 produced constructs capable of producing functional Skp1; that is, Y555 cells transformed with either pGAP24-Skp1 or pGAL14-Skp1 were capable of growth at 37°C, a temperature at which the ts Skp1-11 protein produced by the host strain is non-functional. Expression vectors lacking the *SKP1* cDNA were incapable of complementing the lethality of the *skp1-11* ts mutation.

c) **Restriction Analysis, SDS-PAGE Analysis and Western Blot Analysis of *In Vitro* Fusion Reactions**

pUNI-Skp1 was recombined with pGst-*lox* (Example 2) using the *in vitro* reaction described in Example 4; 0.2 µg of Gst-Cre was used per 20 µl reaction. The resulting plasmid fusion was termed pGST-Skp1. Figure 9A provides a schematic showing the starting constructs and the predicted fusion construct. Five microliters of the fusion reaction mixture was used transform DH5α cells as described in Example 4. The transformed cells were plated onto LB/Amp/Kan plates and plasmid DNA was isolated from individual Ap^RKn^R colonies. The plasmid DNAs were digested with *Pst*I followed by electrophoresis on agarose gels to examine the structure of the fused plasmids. A representative ethidium bromide-stained gel is shown in Figure 9B. In Figure 9B, lane "M" contains DNA size markers, lanes pUNI-Skp1 and pGst-*lox* contain the starting plasmids digested with *Pst*I and lanes 1-12 contain plasmid DNA from individual Ap^RKn^R colonies digested with *Pst*I. Lanes marked with an "*" indicate that these colonies contained a trimeric fusion plasmid that resulted from the fusion of two Gst-*lox* plasmids and one pUNI-Skp1 plasmid. The sizes of the two *Pst*I fragments which result from the fusion of pUNI-Skp1 and pGst-*lox* in kb are indicated (5.8 and 2.0 kb). The results shown in Figure 9B demonstrate that the *in vitro* fusion reaction resulted in the production of the desired fused construct with high efficiency (about 83% of the plasmids in the Ap^RKn^R colonies comprised the fusion of one pUNI-Skp1 vector with one pGst-*lox* vector).

Three individual Ap^RKn^R colonies were picked and grown in liquid cultures which were induced with IPTG to examine whether the fused construct (pGst-Skp1) could produce the desired Gst-Skp1 fusion protein. The cultures were grown, induced and cell extracts were prepared as described in Example 6. An aliquot of the cell lysates prepared from induced and uninduced cells were electrophoresed on an SDS-PAGE gel and the gel was either stained with Coomaise blue or transferred to nitrocellulose to generate a Western blot. The Western blot was probed using an anti-

Skp1 polyclonal antibody (the antibody was raised against the yeast Skp1 using conventional methods). The resulting Coomassie-stained gel and Western blot are shown in Figures 10A and 10B, respectively.

In Figure 10A, lane "M" contains protein molecular weight markers (size in kd is indicated). Lanes marked "C" contain extracts prepared from *E. coli* containing a GST-SK_{P1} construct made by conventional cloning (*i.e.*, the SK_{P1} cDNA was excised using restriction enzymes and inserted into pGEX-2TKcs (Example 2)). Lanes 1-3 contain extracts from Ap^RKn^R cells transformed with *in vitro* fusion reaction mixtures. Extracts prepared from uninduced cells and IPTG induced cells are indicated by "-" and "+", respectively. The arrowheads indicate the location of the Gst-Skp1 fusion proteins. The Gst-Skp1 fusion product generated from the pGST-SK_{P1} fusion construct contains 15 additional amino acids which are located between the Gst domain and the Skp1 protein sequences relative to the Gst-Skp1 fusion protein expressed from the conventionally constructed GST-SK_{P1} plasmid (the additional 15 amino acids are encoded by the linker comprising the loxP site; see Figure 3). In Figure 10B, the lane designations are the same as described for Figure 10A. This Western blot confirms that the bands indicated by the arrowheads in Figure 10A represent Gst-Skp1 fusion proteins.

The results shown in Figures 10A and 10B demonstrate that the Univector Fusion System can be used to create an expression vector that maintains the proper translational reading frame and permits the expression of a fusion protein comprising the expression vector-encoded affinity tag and the protein of interest.

The above results demonstrate that the Univector Fusion System can be used to recombine two plasmids, one containing a gene of interest but no promoter (this vector may optionally contain expression signals such as termination signals and/or polyadenylation signals) and the other containing a promoter and optionally other expression signals (*e.g.*, splicing signals, translation initiation codons) (and optionally sequences encoding an affinity domain) but lacking a gene of interest, *in vitro* in such

a manner that the proper translational reading frame is maintained permitting the expression of a functional protein from the fused plasmids in the host cell.

d) Additional Examples

The *S. cerevisiae SKP1* ORF (Bai *et al.*, *supra*) in pUNI-10 was fused to the pGST-*lox* host vector pHB2-GST by UPS to create a bacterial Gst-lox-Skp1 fusion protein expressed under the control of the *E. coli tac* promoter. A similar Gst-Skp1 expression plasmid lacking *loxP* (*i.e.*, pCB149) made by conventional cloning, was used as a control. Approximately equal amounts of the two fusion proteins were expressed as shown in Figure 16A and B, indicating that the presence of *loxP* did not significantly affect either the transcription or translation of the fusion protein. In this figure, proteins were separated by SDS-PAGE and stained with Coomassie blue (Figure 16A) or immunoblotted (Figure 16B) with anti-Skp1 antibodies. Protein from a control GST-Skp1 expression plasmid lacking *loxP* (lanes 1 and 2) and three independent transformants of UPS-derived Gst-*lox*-Skp1 expression constructs (lanes 3-8) are shown. The asterisk denotes a degradation product.

In another example, to measure the effect of the *loxP* sequence upon eukaryotic expression in the context of transcriptional fusions, the *SKP1* ORF was placed under the control of the *S. cerevisiae GAL1* promoter both by conventional means and by UPS. In this case, it was observed that the relative expression level of the UPS-derived plasmid was slightly lower. This reduction in expression might be explained by the ability of *loxP* RNA to form a 13 bp stem-loop, as secondary structures formed within the 5' UTR of an mRNA can interfere with the initiation of translation [Kozak (1989) *Mol. Cell. Biol.* 9:5134], although an understanding of the mechanism is not required to practice the present invention, and the present invention is not limited to any particular mechanistic explanation. To test this hypothesis, a series of *lox* sites were made containing mutations designed to reduce the stability of the stem-loop, as described in Example 8.

In yet other examples, multiple genes have been tested using UPS and expressed in several different organisms. In addition to Gst-Skp1 expression in bacteria, Myc-Rnr4 and Myc-Rad53 have been expressed in *S. cerevisiae* as shown in Figure 17, showing a comparison of expression levels between *loxP* and *loxH* containing constructs. Protein extracts were prepared from Y80 cells grown in SC-ura plus galactose containing the following plasmids: vector alone (lane 1), pMH176 (*GAL-MYC3-RNR4*) made by conventional cloning lacking a *lox* sequence (lane 2), UPS-derived *GAL-lox-MYC3-RNR4* constructs with either *loxP* (lane 3) or *loxH* (lane 4) present between the *GAL1* promoter and the *MYC3-RNR4* gene, vector alone (lane 5), and UPS-derived *GAL1-MYC3-lox-RAD53* construct (lane 6). The recipient vector for RAD53 was pHY314-MYC3.

Furthermore, many baculovirus expression constructs have been made by UPS and tested. Shown in Figure 18, as illustrative examples, are Gst-Rad53, Myc-Rad53, and HA-Rad53. For Rad53, the UPS-derived constructs express at the same level as Gst-Rad53 made by conventional methods (Figure 18, compare lanes 1 and 2). Figure 18 shows the expression of the UPS-derived baculovirus expression constructs in insect cells. UPS reactions were performed between pUNI-10-RAD53 clones and baculovirus expression vectors in pVL1392 backbones engineered to contain *lox* sites and epitope tags. Host insect expression vectors used were pH100-GST, pH100-MYC3, and pH100-HA3 and the resulting fusion plasmids were crossed onto Baculogold (Pharmingen) by standard methods. GST affinity purified protein from lysates from 1 million cells infected with baculovirus expressing either GST-RAD53 made by conventional cloning (lane 1) or UPS (lane 2) were fractionated on a SDS-PAGE and Coomassie stained. Western blots of protein prepared from cells infected with the baculoviruses containing vector alone (lane 3), UPS-derived *MYC3-lox-RAD53* (lane 4), vector alone (lane 5), or UPS-derived *HA3-lox-RAD53* (lane 6) were probed with anti-Myc (lanes 3-4) or anti-HA (lane 5-6) monoclonal antibodies.

In yet other examples, in mammals, the present invention demonstrated expression of a Myc-tagged F-box protein under the control of the CMV promoter

when transfected into Hela cells as shown in Figure 19. This figure shows immunoblotting of whole cell lysates with anti-HA antibodies. The cells used were Hela cells transfected by the calcium phosphate method with the CMV expression vectors pHM200-HA3 or pHM200-HA3-F3, expressing an HA-tagged F-box protein.

5 In all, over 200 UPS derived constructs have been made and tested, showing expression success rates indistinguishable from those of conventional cloning methods.

EXAMPLE 6

Construction Of An *E. coli* Strain That Inducibly Expresses Cre Recombinase

An *E. coli* strain containing a *cre* gene under the control of an inducible promoter, termed the QLB4 strain, was constructed as follows. The *cre* gene was placed under the transcriptional control of the inducible *lac* promoter by inserting the *cre* ORF into a derivative of pNN402 [Elledge *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:1731]; pNN402 was modified to contain a *lac* promoter. This construct was then crossed onto lambda phage (e.g., λgt11) using conventional techniques. The recombinant lambda phage carrying the *lac-cre* gene was integrated into the chromosome of *E. coli* strain JM107 to generate the QLB4 strain.

Expression of Cre recombinase was induced by growing QLB4 cells at 37°C until an OD₆₀₀ of 0.6 was reached. The culture was then split into 2 parts and IPTG was added to one part to a final concentration of 0.4 mM. As a control, the BNN132 strain (ATCC 47059; Elledge *et al.* (1991), *supra*) which contains the *cre* gene under the transcriptional control of the endogenous *cre* promoter was treated as described for the QLB4 strain. Cell extracts (total protein) were prepared from all four samples (QLB4 ± IPTG and BNN132 ± IPTG) and examined for expression of Cre recombinase by Western blotting analysis. The Western blot was probed using a rabbit polyclonal anti-Cre antibody (Novagen) as the primary antibody and a goat anti-rabbit IgG horseradish peroxidase conjugate (Amersham) as the secondary antibody according to the manufacturer's instructions. Figure 11 shows a Western blot containing extracts

prepared from (shown left to right) BNN123 cells grown in the absence of IPTG ("C") and QLB4 cells grown in the absence ("QLB4 -") and presence of IPTG ("QLB4 +"), respectively. The location of the Cre recombinase band is indicated by the arrowhead. The additional bands seen on this Western blot are due to cross-reactivity of the crude (i.e., not affinity purified) rabbit anti-Cre antibody with bacterial proteins.

Western blot analysis demonstrated that Cre protein could not be detected in BNN123 cells grown in the presence or absence of IPTG. Cre protein was detected in QLB4 cells grown in the presence of IPTG, but not in the absence of IPTG, by Western blot analysis. Therefore, the expression of Cre recombinase in QLB4 cells is greatly induced by the presence of IPTG in the growth medium. By this analysis, the expression of Cre recombinase in QLB4 cells is dependent upon the induction of the *lac-cre* gene by IPTG. However, more sensitive functional assays indicate that the Cre protein was expressed constitutively at very low levels in both BNN132 cells and QLB4 cells in the absence of IPTG. In these functional assays, a pUNI vector (Kn^R) and a pHOST vector (Ap^R) were cotransformed into QLB4 cells and the transformed cells were grown on plates containing kanamycin to select for the presence of the pUNI-pHOST fusion plasmid. Plasmid DNA was isolated from individual kanamycin-resistant colonies and subjected to restriction enzyme digestion to examine the structure of the plasmid DNA. This analysis revealed that multiple isoforms of the plasmid fusion product were present in the plasmid DNA isolated from any single kanamycin-resistant colony. While not limiting the present invention to any particular mechanism, it is believed that low level constitutive expression of Cre recombinase leads to multiple fusion events between the pUNI and pHOST vectors resulting in the production of multimeric forms (i.e., trimer, tetramer, etc.) of the fused plasmid (the desired fused plasmid is a dimer formed by fusion of pUNI and pHOST). The multimeric plasmid fusion products would be expected to be unstable due to the fact that the Cre protein is constitutively expressed in QLB4 cells.

To overcome the potential problems that low level constitutive expression of the *cre* gene in the host cell may cause, the expression of *cre* can be more tightly controlled as described below. In addition to the approaches described below, the

pUNI and pHOST vectors can be modified as described in Example 7 and these modified vectors can be fused using a host cell that constitutively expresses the Cre protein.

The expression of Cre recombinase can be more tightly controlled by a variety
5 of means. For example, the expression of the *cre* gene can be made conditional when expressing *cre* under the control of the *lac* promoter by growing the host cells in medium containing glucose. The presence of 0.2% glucose in the growth medium virtually shuts down transcription from the *lac* promoter. In addition, the *lac* promoter can be modified to insert additional operator (*o*) sites which bind the *lac* repressor.
10 Other tightly controlled promoters are known to the art (e.g., the T7 promoter which requires the expression of T7 RNA polymerase; these promoters are available on the pET vectors (Novagen)) and may be employed to control the expression of the *cre* gene.

In addition to placing the *cre* ORF under the control of a tightly controlled
15 promoter, Cre expression can be tightly controlled by placing the *cre* gene on a plasmid containing a temperature-sensitive (ts) replicon (e.g., rep pSC101^{ts}). When the *cre* gene is carried on a ts replication plasmid, Cre will be expressed during the transformation of the host cell (because the host cell containing the ts plasmid containing the *cre* gene was maintained at the permissive temperature) but will be
20 absent following recombination of the pUNI and pHOST vectors when the host cell is grown at a temperature non-permissive for replication of the ts replicon.

EXAMPLE 7

In Vivo Recombination In Prokaryotic Hosts Using The Univector Fusion System

As discussed above, Cre-*loxP*-mediated plasmid fusion can occur *in vivo*,
25 although the reverse reaction, resolution of heterodimers, might decrease its utility. Ideally, it would be desirable to have Cre present only transiently to catalyze the initial fusion event, then absent to allow the stable propagation of the recombinant products.

Therefore, a model was tested whereby UPS was explored *in vivo* in the *E. coli* strain BUN13 that conditionally expresses Cre recombinase under *lac* control and in a second strain carrying *cre* on a plasmid, pQL269, with a Ts origin of replication derived from pSC101. Experiments using BUN13 and co-transformation of pUNI-10 and pQL103, an Ap^r*loxP* containing plasmid, showed that the UPS reaction occurred efficiently, but many colonies had a mixture of plasmids that required retransformation into non-cre-expressing strain to stabilize. However, results with the Ts plasmid were better. Competent cells were prepared from JM107/pQL269 cells grown at 42°C for several hours to cause loss of pQL269. Co-transformation of pUNI-10 and pQL103 into these cells followed by selection on kanamycin plates at 42°C revealed that 25% contained the desired single pUNI-10-pQL103 co-integrand. These two experiments demonstrated that UPS can be used to generate plasmid fusions *in vivo* and provide an alternative to the *in vitro* reaction when Gst-Cre is not available.

As described in Example 6 and the experiments above, cotransformation of *E. coli* cells expressing Cre protein (*e.g.*, QLB4, BNN132) with a pUNI construct and a pHOST construct (each construct containing a single *lox* site) results in the fusion of these two constructs *in vivo*. If the host cell used for the recombination reaction constitutively expresses the Cre protein, multimeric forms of the fused constructs are generated. In addition to the methods outlined above for tightly regulating the expression of the *cre* gene in the host cell, cells constitutively producing Cre protein can be employed with modified pUNI and pHOST vectors as described in this example. The pUNI construct is modified such that two different *lox* sites flank the kanamycin resistance gene (the modified pUNI construct is termed pUNI-D). The two *lox* sites differ in their spacer regions by one or two nucleotides and for the sake of discussion the two different *lox* sites are referred to as "*loxA*" and "*loxB*" (*e.g.*, *loxP* and *loxP511*; "*loxB*" is used in this discussion to distinguish it from the first *lox* site termed "*loxA*" and does not indicate the use of the *loxB* sequence found in the *E. coli* chromosome). Cre cannot efficiently catalyze a recombination event between a *loxA* site and a *loxB* due to the sequence changes located in the spacer regions between the

Cre binding sites; however Cre can efficiently catalyze the recombination between two *loxA* sites or two *loxB* sites [Hoess *et al.* (1986) *Nucleic Acids Res.* 14:2287]. The pHOST construct is modified such that one *loxA* site and one *loxB* site flank the selectable marker gene (the modified pHOST construct is termed pHOST-D). In this example, pHOST contains the *sacB* gene as the selectable marker (a negative selectable marker). The presence of the *sacB* gene on pHOST-D provides a means of counter-selection as cells expressing the *sacB* gene are killed when the cell is grown in medium containing 5% sucrose [Gay *et al.* (1985) *J. Bacteriol.* 164:918 and (1983) *J. Bacteriol.* 153:1424].

Figure 12 provides a schematic showing the strategy for *in vivo* recombination in a Cre-expressing host cell (*e.g.*, QLB4 cells) using the pUNI-D and pHOST-D constructs. Arrows are used to indicate the direction of transcription of various genes or gene segments in Figure 12. In Figure 12, the following abbreviations are used: Ap^R (ampicillin resistance gene); Kn^R (kanamycin resistance gene); Ori (non-conditional plasmid origin of replication); Ori^R (the R6K γ conditional origin of replication); Cre (Cre recombinase); GENEX (gene of interest). The strategy outlined in Figure 12 is referred to as the "*in vivo* gene-trap." Figure 12 illustrates that the second *lox* site (*loxB*) in pUNI-D (relative to the design of the pUNI-10 vector) is inserted between the kanamycin resistance gene and the R6K γ conditional origin of replication.

To generate a pHOST-D construct, a commercially available expression vector containing the desired promoter (and optionally enhancer) is modified as described in Example 2 to insert the *loxA* site downstream of the promoter. However, it is not necessary that a commercially available expression vector be employed as the art is well aware of methods for the generation of expression vectors. Sequences encoding the *sacB* gene [Gay *et al.* (1983) *J. Bacteriol.* 153:1424; GenBank Accession Nos. X02730 and K01987] and the second *lox* site (*loxB*) are inserted downstream of the first *lox* site (*loxA*).

The pUNI-D and pHOST-D constructs are cotransformed into QLB4 cells (Example 6) and the transformed cells are plated onto LB/Ap/Kn plates containing 5% sucrose to select for the desired recombinant. Figure 12 illustrates the recombination events that will occur in the presence of Cre in the QLB4 cells. First pUNI-D and pHOST-D will fuse to form two dimers in which two possible double cross-over events can occur. These two double cross-over events are diagrammed in Fig 12. The double cross-over events will result in the exchange of the DNA segments that are flanked by *loxA* and *loxB* to produce the plasmids labelled "A" and "B." All plasmids that contain the *sacB* gene (the pHOST-D, the fused plasmids and plasmid B) will be selected against by the presence of sucrose in the growth medium. The pUNI-D construct will not be able to replicate in QLB4 cells as these cells do not express the Π protein required for replication of the R6K_y origin. Therefore, the only construct that will be maintained in QLB4 cells selected on LB/Kn containing sucrose is the desired plasmid A in which the gene of interest from pUNI-D has been placed under the transcriptional control of the promoter located on pHOST-D.

To illustrate this method, pUNI-10 was modified to place a second *lox* site, comprising the *loxP511* sequence (SEQ ID NO:16) between the kanamycin resistance gene and the R6K_y conditional origin of replication to create pUNI-10-D. A second *lox* site, comprising the *loxP511* site, was inserted onto a *loxP*-containing expression plasmid (*i.e.*, a pHOST vector) to create a pHOST-D vector. One-half of one microgram of each plasmid was cotransformed into competent QLB4 cells and an aliquot of the transformed cells were plated onto LB/Ap plates and onto LB/Ap/Kn plates containing 5% sucrose and the number of colonies on each type of plate were counted. The percentage of Ap^RKn^R colonies which grew on sucrose-containing plates relative to the number of Ap^R colonies was 1% ($1 \times 10^3 / 1 \times 10^5$). Restriction enzyme digestion of plasmid DNA isolated from individual Ap^RKn^R colonies which grew on sucrose-containing plates confirmed that the desired fusions had been generated. These results indicate that the *in vivo* gene trap method can be used to recombine a gene of

interest carried on a pUNI-D vector into an expression vector using host cells that constitutively express the Cre protein.

In addition to providing a means for recombining a gene of interest carried on a pUNI-D vector into an expression vector using host cells that constitutively express the Cre protein, the *in vivo* gene trap method provides a means to transfer a gene of interest contained on a linear DNA molecule (*e.g.*, a PCR product) that lacks a selectable marker into an expression vector(s). The desired PCR product is amplified using two primers, each of which encode a different *lox* site (a "*loxA*" and "*loxB*" site such as a *loxP* and *loxP511* site). A pUNI vector is constructed that contains (5' to 3') 5 a *loxA* site, a counter-selectable marker such as the *sacB* gene and a *loxB* site (*i.e.*, the two different *lox* sites flank the counter-selectable marker). This pUNI vector also 10 contains a conditional origin of replication and an antibiotic resistance gene as described above and in Example 1. The PCR product (*loxA*-amplified sequence-*loxB*) is recombined with the modified pUNI vector (which comprises *loxA*-counter- 15 selectable marker-*loxB*) to create a pUNI vector containing the PCR product which now lacks the counter-selectable marker. This recombination event is selected for by growing the host cells in medium that kills the host if the counter-selectable gene is expressed. The PCR product in the pUNI vector (containing 2 *lox* sites) can then be placed under the control of the desired promoter element by recombining the 20 pUNI/PCR product construct with the appropriate pHOST-D vector.

EXAMPLE 8

The Use Of Modified *LoxP* Sites To Increase Expression Of The Protein Of Interest

The pUNI and pHOST constructs employed in the Univector Plasmid Fusion System were designed such that plasmid fusion resulted in the introduction of a *lox* site between the promoter and the gene of interest. *LoxP* sites consist of two 13 bp 25 inverted repeats separated by an 8 bp spacer region [Hoess *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79:3398 and U.S. Patent No. 4,959,317]. Transcripts of the gene of

interest produced from a pUNI-pHOST fusion construct comprising a *loxP* site may have two 13 nucleotide perfect inverted repeats within the 5' untranslated region (UTR) that have the potential to form a stem-loop structure (this will occur in those cases where pHOST does not encode an affinity domain at the amino-terminus of the fusion protein). It is currently believed that the ribosome scanning mechanism is the most commonly used mechanism for initiation of translation in eukaryotes (e.g., yeast and mammalian cells). Using this mechanism, the ribosome binds to the 5' cap structure of the mRNA transcript and scans downstream along the 5' UTR searching for the first ATG or translation start codon. Without limiting the present invention to any particular mechanism, it is possible that a stem-loop structure formed by the presence of a *loxP* sequence on the 5' UTR of the mRNA encoding the protein of interest would block or reduce the efficiency of ribosome scanning and thus the translation initiation step could be impaired. There is evidence that stem-loop structures in the 5' UTR of particular mRNAs reduce the efficiency of translation in eukaryotes [see, e.g., Donahue *et al.* (1988) *Mol. Cell. Biol.* 8:2964 and Yoon *et al.* *Genes and Dev.* (1992) 6:2463]. It is noted that no evidence suggests that the presence of a stem-loop structure in the coding region (as opposed to the 5' UTR) of a transcript negatively affects its ability to be translated. It is likely that the energy of protein synthesis is sufficient to overcome secondary structures present in mRNAs. Indeed the data presented in Example 5 shows that a GST-SKPI fusion construct produced using the Univector Fusion System (*i.e.*, the construct contains a *loxP* site between the sequences encoding the Gst and Skp1 domains) produced the same level of fusion protein as did a conventional construct encoding a Gst-Skp1 fusion protein which lacks the *loxP* sequence. Therefore, concerns over the presence of a stem-loop structure caused by the presence of a *lox* sequence in a transcript encoded by a pUNI-pHOST fusion construct are limited to those constructs that do not generate fusion proteins.

If low levels of expression are observed when a gene of interest is expressed from a pUNI-pHOST fusion constructs comprising *lox* sequences that comprise perfect

13 bp inverted repeats (*e.g.*, *loxP*), pUNI and pHOST constructs containing mutated
5 *loxP* sequences are employed. The mutated *loxP* sequences comprise point mutations
that create mismatches between the two 13 bp inverted repeat sequences within the
loxP site that disrupt the formation of or reduce the stability of a stem loop structure.
10 Specifically, two modified *loxP* sites were designed that have mismatches at different
positions in the inverted repeats located within a *loxP* site. The 13 bp inverted repeats
are binding sites for the Cre protein; thus, each *loxP* site has two binding sites for Cre.
For the purpose of discussion, these two binding sites are referred to as L and R (left
and right). The wild-type *loxP* site is designed L(0)-R(0) wherein "0" indicates the
15 absence of a mutation (*i.e.*, the wild-type sequence). Two derivatives of the wild-type
loxP sequence were designed and termed *loxP2* and *loxP3*. The sequence of *loxP2*
(SEQ ID NO:13), *loxP3* (SEQ ID NO:14), as well as the wild-type *loxP* sequence
(SEQ ID NO:12) are shown in Figure 13. *LoxP2* is placed on the pUNI-10 construct
(in place of the wild-type *loxP* site) and *loxP3* is placed on the pHOST construct.

15 *LoxP2* has repeats designated L(3,6)-R(0) which indicates that the third and
sixth nucleotides of the left repeat are mutated; thus, a mismatch is introduced at the
third and sixth positions between the L and R repeats of the *loxP2* site. *LoxP3* has
repeats designated L(0)-R(9) which indicates that the ninth nucleotide on the right
repeat sequence is mutated to introduce a mismatch at the ninth position between the L
20 and R repeats of the *loxP3* site. Fusion between the *loxP2* site on the pUNI construct
and the *loxP3* site on the pHOST construct will generate a hybrid *loxP23* site [L(3,6)-
R(9)] located between the promoter and the gene of interest and a wild-type *loxP* site
[L(0)-R(0)] at the distal junction. Thus, the *loxP23* site (SEQ ID NO:15) in the 5'
25 UTR will have three mismatches distributed at positions 3, 6 and 9 between the 13
nucleotide inverted repeats which are expected to strongly destabilize the formation of
the stem-loop structure. Other mutated *loxP* sequences suitable for disruption of the
stem-loop structure will be apparent to those skilled in the art; therefore, the present
invention is not limited to the use of the *loxP2* and *loxP3* sequences for the purpose of
disrupting stem-loop formation on the 5' UTR of transcripts produced from pUNI-

pHOST fusion constructs. The suitability of any pair of mutated *lox* sites for use in the Univector Fusion system may be tested by placing one member of the pair on a pUNI vector and the other member on a pHOST construct. The two modified vectors are then recombined *in vitro* as described in Example 4 and the fusion reaction mixture 5 is used to transform *E. coli* cells and the transformed cells are plated on selective medium (*e.g.*, on LB/Amp and LB/Kan plates) in order to determine the efficiency of recombination between the two mutated *lox* sites (Example 4). The efficiency of recombination between the two mutated *lox* sites is compared to the efficiency of recombination between two wild-type *loxP* sites. Any pair of two different mutant *lox* 10 sites that recombines at a rate that is about 5% or greater than that observed using two *loxP* sites is a useful pair of mutated *lox* sites for use in avoiding the formation of a stem-loop structure on the 5' UTR of the mRNA transcribed from the pUNI/pHOST fusion construct.

A strategy as described above was employed to determine if the reduced 15 expression observed with the *SKP1* ORF under control of the *GAL1* promoter as described in Example 5 could be improved with mutated *lox* sites. A series of *lox* sites designed to reduce the stability of the stem-loop were employed. These, together with a control scrambled site, *loxS*, were placed between the *GAL1* promoter and the *lacZ* reporter gene and β-galactosidase expression was measured. Mutations that 20 decreased stem-loop stability tended to express better and one mutant, *loxP^{L369}*, did not display any inhibitory effects. This mutant also retained 25% of the wild-type recombination efficiency and has been designated *loxH* (*i.e.*, for host). The oligonucleotides used to generate the *loxH* site are based on the *loxH* sequence 5'-ATTACCTCATATAGCATACATTACGAAGTTAT-3' (SEQ ID NO:32). *LoxH* 25 was further tested by using it to place *MYC-RNR4* under *GAL1* control and showed no translational interference, as shown in Figure 17 (compare lanes 2, 3, and 4). *LoxH*'s 25% recombinational efficiency is well within the range useful for UPS-mediated plasmid constructions. Thus, it is recommended that *loxH* be used in pHOST recipient

vectors intended for transcriptional fusions to maximize expression, while *loxP* should be used for all other applications because of its higher recombination efficiency.

It will be apparent to those skilled in the art that a similar strategy can be employed for the modification of *frt* sites when the FLP recombinase is employed for the recombination event. The *frt* site, like *lox* sites, contains two 13 bp inverted repeats separated by an 8 bp spacer region.

EXAMPLE 9

Precise ORF Transfer (POT)

In order to transfer only the gene of interest from the Univector to the Host vector, the present invention provides a second recombination event that allows a resolution of the UPS generated heterodimer. A schematic representation of the POT reaction is shown in Figure 20. In one embodiment of the present invention, a R-recombination site, RS, was placed after the cloning site in pUNI (*i.e.*, pUNI-20) such that any gene inserted into pUNI-20 would be flanked on the 5' side by *loxP* and on the 3' side by RS, although the present invention contemplates the use of any other second recombination system (*e.g.*, the Res system). Host recipient vectors must also contain *lox* and RS elements in the correct order. The initial fusion event is catalyzed by Cre by UPS. The second reaction can be catalyzed *in vitro* by incubation with purified R-recombinase (Araki *et al.*, *supra*) or *in vivo* by transformation into a strain (*e.g.*, BUN15) expressing the R-recombinase under *tac* control on a Ts replication plasmid (*e.g.*, pML66) that is lost when cells are plated at 42°C. POT works efficiently as a two step reaction *in vivo* or *in vitro*. Efficient resolution *in vivo* without a selection for the second recombination event requires incubation in LB plus IPTG after transformation prior to plating on selective media. An incubation of 1 h and 4 h gave 3% and 15% recombinants, respectively, which showed complete loss of the pUNI backbone through recombination between RS sequences. *In vitro* recombination catalyzed by the R recombinase achieved 30% recombinants.

The efficiency of recovering plasmids that have undergone POT can be greatly enhanced through the use of a recipient vector in which a counter-selectable marker is placed between the *loxP* and RS sites. For this purpose, the present invention utilized the Φ X174 E gene which is toxic when expressed in *E. coli* unless the host cell lacks the *slyD* gene [Maratea *et al.* (1985) *Gene* 40:39]. pAS2-E, a two hybrid bait vector derived from pAS2 [Durfee *et al.* (1994) *Gene. & Dev.* 7:555] which contains in a 5' to 3' order *loxP*, E under control of the *tac* promoter, and an RS site, was fused with pUNI-20, containing the *SKP1* gene and the co-integrand was selected by transformation into CX1 (*slyD*⁻). This co-integrand was then transformed into BUN15 cells expressing the R recombinase and resolution events were isolated by selecting for Ap^r in the presence of IPTG to induce the E protein. Since BUN15 is *slyD*⁺, pAS2-E alone cannot survive in it because of toxicity due to E expression. However, when pAS2-E is fused to pUNI-20 derivatives, it can transform that strain because subsequent R-dependent site-specific recombination between RS sites will eliminate both the pUNI backbone and E. This results in the replacement of E with the corresponding region from pUNI. One hundred percent (24 of 24) Ap^r transformants resulting from the transformation of the pAS2-E-pUNI-20-SKP1 fusion plasmid showed precise transfer of the *SKP1* gene from pUNI-20 into pAS2-E with only 1 hr incubation prior to plating on selective media.

Transformation of a heterodimeric plasmid with E flanked by RS sites into BUN15 gave a transformation several orders of magnitude greater than transformation of the pAS2-E plasmid itself. This demonstrated that POT can be achieved in a single step by direct transformation of a UPS reaction into BUN15 (*i.e.*, rather than a two-step process). pUNI-20-SKP1 and pAS2-E were incubated with Gst-Cre in a standard UPS reaction and the reaction mixture was transformed directly into BUN15 and AP^r transformants were selected at 42°C after an hour incubation. One hundred percent (20 of 20) of Ap^r transformants were found to have undergone POT with *SKP1* replacing the E gene in pAS2-E as determined by restriction digestion with *PvuII*, as shown in Figure 21. The sample shown in Figure 21 was generated from plasmid DNA isolated

from 10 different Ap^r transformants, digested as described above along with two parental plasmids, P1 (pUNI-20-SKP1) and P2 (pAS2-E) and I (the UPS generated pUNI-20-SKP1-pAS2-E recombination intermediate). Precise ORF transfer resulted in the generation of a novel 800 bp *PvuII* fragment indicated by the arrowhead.

5 For POT assays, BUN15 cells were grown overnight in LB containing spectinomycin (50 µg/ml) at 30°C. BUN15 cells were diluted 1 to 100 in fresh media LB/Spec media containing 0.3 mM IPTG and grown to OD of 0.5. Electrocompetent cells were prepared as recommended (Biorad). Forty µl of competent cells were used in each transformation. After the electrotransformation, cells were incubated in LB
10 plus IPTG for 1-8 hr for recovery before being plated on LB/Amp/IPTG 1mM and incubated at 42°C.

EXAMPLE 10

Library Transfer Using UPS

The ability to use the methods and compositions of the present invention for generating and subcloning entire nucleic acid libraries is demonstrated in this Example.
15 A random shear *S. cerevisiae* genomic library was made in pUNI-10 using the *XbaI*- adaptor strategy [Elledge *et al.* (1991) *Proc. Natl. Acad. Sci.* 88:1731]. This library had 5x10⁵ recombinants with 80% inserts ranging from 3 kb to 8 kb. This library was fused to pRS425-*lox*, a URA3 2µ plasmid, using UPS and 1.6x10⁶ recombinant fusion
20 plasmids were recovered. This library was used to transform an *S. cerevisiae cdc4-1* mutant strain Y543 and Ura⁺ transformants were selected at 34°C, the non-permissive temperature of *cdc4-1*. Of 31 plasmids capable of conferring growth at 34°C, three classes were recovered. One class was *CDC4* as expected, the second was *SKP1*, and the third was *CLB3*. *SKP1* and *CLB4*, a cyclin closely related to *CLB3*, had been
25 previously shown to suppress *cdc4-1* mutants when overexpressed from the *GAL* promoter [Bai *et al.* (1994) *EMBO J.* 3:6087; and Bai *et al.*, *supra*]. These experiments demonstrate the feasibility of library transfer using UPS. In cases where a

cDNA expression library is created, such as for the two hybrid system, once clones have been isolated, they can be rapidly converted back into simple Univector clones by Cre recombination *in vivo*. Using UPS, these plasmids can now be rapidly fused with any of a series of pHHOST expression vectors for future analytical needs.

5

EXAMPLE 11

General Material and Methods

This Example provides general materials and methods used throughout the experiments discussed above and below.

I. Media, Enzymes, and Chemicals

10 For drug selections, LB plates or liquid media were supplemented with either kanamycin (40 µg/ml) or ampicillin (100 µg/ml). When necessary, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.3 mM and X-Gal (Sigma) was used at 80 µg/ml. Yeast growth media and plates were made according to Rose *et al.* [Rose *et al.* (1990) *Laboratory course manual for methods in yeast genetics*, Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press].
15 Restriction endonucleases, large (klenow) fragment of *E. coli* DNA polymerase I, T4 polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase were purchased from New England Biolabs. Drugs were purchased from Sigma if not otherwise specified.

II. Bacterial and Yeast Strains

20 *E.coli* BW23474 [Δlac -169, *robA1*, *creC510*, *hsdR514*, *uidA*($\Delta MluI$)::*pir*-116, *endA*, *recA1*] and BW23473 [Δlac -169, *robA1*, *creC510*, *hsdR514*, *uidA*($\Delta MluI$)::*pir*⁺, *endA*, *recA1*] (Metcalf *et al.*, *supra*) was a gift of B. Wanner and was used as host for propagation of all Univector based plasmids. BUN10 [*hisG4 thr-1 leuB6 t lacY1 kdgK51* $\Delta(gpt-proA)$ 62 *rpsL31 tsx33 supE44 recB21 recC22 sbcA23 hsdR::cat-pir-116(Cm^R)*] was used for homologous recombination experiments. BUN13 which has

cre under the control of the *lac* promoter is JM107 lysogenized with λ_{LC} (*aadA lac-cre*). BUN15 is XL1 blue containing pML66(*tac-R, SP'*) and was used for the *in vivo* RS recombination assays. *E. coli* JM107 or DH5 α [Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Lab., Cold Spring Harbor, NY, 2nd Ed.] were the transformation recipients for all other plasmid construction, including those made by UPS. *E. coli* BL21 was used as the host for bacterial expression studies. CX1 (*ara leu purE gal trp his argG rpsL thi-1 supE lacI^Q slyD1*) was used for propagation of *E* expression clones. *S. cerevisiae* Y80 [Zhou and Elledge (1992) *Genetics* 131:851] was used for yeast expression studies and Y543 (as Y80 but *cdc4-1*) was used for *cdc4* suppression (Bai *et al.*, 1994, *supra*).

III. Plasmid Construction

The construction of several of the plasmids used in the examples of the present invention are provided below. These examples are provided to illustrate strategies and general methods used in making plasmids for use in the UPS. However, these specific plasmids and methods of construction are not required to practice the present invention.

For the Gst-Cre expression construct, pQL123, the *cre* ORF was amplified by PCR and an *NcoI* site placed at the first ATG using primers 5'-CCATGGCCAATTACTGACCGTACAC-3' (SEQ ID NO:21) and 5'-CCCGGGCTAACCGCCATCTTCCAGC-3' (SEQ ID NO:20). The PCR product was cloned into pCRTMII (Invitrogen) and subcloned as a *NcoI-EcoRI* fragment into *NcoI-EcoRI* digested pGEX-2Tkcs to create pQL123.

The pHOST plasmid pQL103 was made by deleting one *loxP* site from pSE1086, which contains a *XhoI-loxP-NotI-loxP-SalI* cassette, by digestion with *NotI* and *SalI*, filling in the ends with klenow and religation. The 590 bp *NcoI-BamHI* fragment containing the *S. cerevisiae SKP1* ORF was subcloned from pCB149 into *NcoI-BamHI*-cut pUNI-10 to create pQL130(pUNI-SKP1).

A second subclone of *SKP1* is pML73 which contains the same 5' end of *SKP1* but an additional 800 bp of genomic DNA to the next *BamHI* site at the 3' end cloned into pUNI-20. pML73 was used for the POT experiments. An oligo linker containing *loxP* and flanked by *NcoI* and *BamHI* overhangs was made by annealing two oligos 5'-
5 CATGGCTATAACTCGTATAGCATACATTATACGAAGTTATG-3' (SEQ ID NO:22) and 5'-GATCCATAACTCGTATAATGTATGCTATACGAAGTTAT-3' (SEQ ID NO:23), and then ligating into *NcoI* and *BamHI* digested pGEX-2TKcs to create pHB2-GST. The *MYC₃-RNR4* gene was subcloned from pMH176 [Huang and Elledge (1997) *Mol. Cell. Biol.* 17:6105] as a *XhoI-SacI* fragment into *XhoI-SacI*-cleaved pUNI-10 to create pQL248, or into *SalI-SacI* digested pBAD104, a *GAL1* expression vector to create the control lacking *loxP*. Two pBAD104 derived recipient vectors, pQL138 and pQL193, were constructed by insertion of either a wild type *loxP* or *loxP³⁶⁹* sequence into the polylinker using primer pairs:

10 5'-TCGAGACGTCATAACTCGTATAGCATACATTATACGAAGTTATGC-3'
15 (SEQ ID NO:24) and
5'-GCCGCATAACTCGTATAATGTATGCTATACGATGTTATGACGTC-3' (SEQ ID NO:25) (pQL138), or
5'-CATGGCTATAACTCGTATAGCATACATTATACGAAGTTATG-3' (SEQ ID NO:26) and
20 5'-GATCCATAACTCGTATAATGTATGCTATACGAAGTTATAGC-3' (SEQ ID NO:27) (pQL193). Two *GAL1:MYC₃-RNR4* constructs were made by UPS between pQL248 and pQL138 or pQL193.

25 For the construction of pQL269 (*lac-cre aadA* on a Ts pSC101 ori), the *EcoRI-PvuII* fragment from pQL114 containing *aadA* and the *lac-cre* gene fusion was ligated to a *BglII* (made blunt by T4 polymerase)-*EcoRI* fragment from pINT-ts [Hasan *et al.* (1994) *Gene* 150:51] containing the Ts replication origin and transformants were screened for Sp^R and Ts growth at 42°C. A plasmid with those properties was designated pQL269.

pML66 was constructed by ligating the *EcoRI-SalI* (blunt) fragment containing the *tac* promoter driving the R recombinase from pNN115 (Araki *et al.*, *supra*) into *EcoRI-PstI* (blunt) cleaved pQL269. This spectinomycin resistant plasmid expresses R protein in the presence of IPTG and is lost from cells grown at 42°C because of a temperature sensitive replication mutation.

pUNI-Amp was made by placing the *bla* gene from pUC19 in place of the *neo* gene on pUNI-20 by generating a PCR product of *bla* and ligating that into *MluI-NheI* (blunt) cleaved pUNI-20. The subcloning of the triple MYC tag into pUNI-Amp was accomplished by PCR amplification of the 3xMYC tag present of pJBN48 by the

primers MZL154, 5'-AAATTTCTCGAGGCTCTGAGCAAAAGCTCAT-3' (SEQ ID NO:28) and MZL155,

5'-TATATATAGCGGCCGCTTAATTAGATCCTCCTCGGATA-3' (SEQ ID NO:29), followed by cleavage of the PCR product with *XhoI* and *NotI* and ligation into *XhoI-NotI* cleaved pUNI-Amp to generate pML74. Sequence of the PCR primers used to amplify the 3xMYC tag from pML74 for tagging the C-terminus of SKP1 by homologous recombination were primer A (MZL160)

5'-CCAGAGGAGGAGGCTGCCATTAGGCGTGAAAATGAATGGGCTGAAGACCG TCTGAGCAAAAGCTCATTTC-3' (SEQ ID NO:30) and primer B (MZL161)

5'-GGATATAGTTCCCTCCTTCAGC (SEQ ID NO:31).

pAS2-E was constructed by first placing a synthetic *loxP* site between the *NcoI-SalI* sites of pAS2 to make pAS2-*lox*, and then generating a *E*-containing fragment with the following features: 5' *XhoI* site, *tac* promoter driving *E*, *SpeI* site 3' and ligated the *XhoI-SpeI* fragment together with a *SpeI-PstI* synthetic RS fragment into *XhoI-PstI* cleaved pAS2-*lox* to make pAS2-E (pML71).

IV. β -galactosidase Assays

Yeast cells expressing the *GAL1:lacZ* reporter constructs containing different *loxP* sequences were grown at 30°C to mid-log phase ($OD_{600} = 0.5\text{-}0.6$) in SC-Ura media containing 2% raffinose, galactose was added to 2% final, and cells were

incubated at 30°C for two hours. β -galactosidase activities were measured as described by Zhou and Elledge (Zhou and Elledge, *supra*).

EXAMPLE 12

Construction of BUN13

5 This Example describes the construction of BUN13, a lambda lysogen with *cre* under *lac* control. pSE356 contains a cassette consisting of the *Tn5 neo* gene, the *lac* promoter, and a polylinker sequence surrounded by stretches of λ DNA sequence.

10 pQL114, the plasmid used to recombine the *cre* gene into λ , was constructed in two steps. First, the *BamHI-HindIII* (made blunt by T4 DNA polymerase) fragment containing the spectinomycin resistance gene *aadA* from pDPT270 [Taylor and Cohen (1979) J. Bacteriol 137:92] was subcloned into *BamHI-SphI* (made blunt by T4 DNA polymerase digested pSE356) to create pQL102, replacing *neo* with *aadA*. Secondly, a *NotI* site was engineered at the 5' end of the ribosomal binding site of the *cre* gene by PCR using primers 5'-GCGGCCGCTGAGTGTAAATGTCCAATT-3' (SEQ ID NO:19) and 5'-CCCGGGCTAACCGCCATCTTCCAGC-3' (SEQ ID NO:20). The

15 PCR product was cloned into pCRTMII and subcloned as a *NotI-EcoRI* fragment into *NotI-EcoRI* digested pQL102 to create pQL114, placing *cre* under *lac* control adjacent to *aadA* and flanked by λ DNA sequence. λ^{KC} (Elledge *et al.*, *supra*) was amplified on JM107 containing pQL114 and the resulting phage lysate containing the desired

20 recombinant λ_{LC} phage was used to infect JM107. Sp^rKn^s lysogens were selected and tested for Cre expression and the ability to perform UPS. One strain with those properties was designated BUN13.

25 It is clear from the above that the present invention provides methods for the subcloning of nucleic acid molecules that permit the rapid transfer of a target nucleic acid sequence (*e.g.*, a gene of interest) from nucleic acid molecule to another *in vitro* or *in vivo* without the need to rely upon restriction enzyme digestions.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

5